UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA



BILIRUBIN DYSREGULATES MYELINATION IN EARLY NEONATAL LIFE

Andreia Pereira Barateiro

DOUTORAMENTO EM FARMÁCIA
(Biologia Celular e Molecular)

2012

UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA



BILIRUBIN DYSREGULATES MYELINATION IN EARLY NEONATAL LIFE

Andreia Pereira Barateiro

Research advisors: Dora Maria Tuna de Oliveira Brites, PhD.
Adelaide Maria Afonso Fernandes Borralho, PhD.

DOUTORAMENTO EM FARMÁCIA
(Biologia Celular e Molecular)

2012

BILIRUBIN DYSREGULATES MYELINATION IN EARLY NEONATAL LIFE

A BILIRRUBINA ALTERA A MIELINIZAÇÃO NO PERÍODO NEONATAL PRECOCE

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (Biologia Celular e Molecular)

Andreia Pereira Barateiro

2012

Para a elaboração da presente tese de doutoramento foram usados integralmente como capítulos, artigos científicos já publicados, submetidos para publicação, ou em preparação para publicação, em revistas científicas internacionais indexadas. Na concepção, elaboração e concretização dos trabalhos participaram igualmente os seguintes autores: Ana Rita Vaz, Sandra Leitão Silva, Veronique Emela Miron, Charles ffrench-Constant, Sofia Duque Santos, Helena Sofia Domingues, João Bettencourt Relvas, Shujuan Chen, Robert Tukey, Adelaide Fernandes e Dora Brites.

De acordo com o disposto no ponto 1 do artigo nº41 do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, deliberação nº 93/2006, publicada em Diário da República – II Série nº 153 – 5 de Julho de 2003, a Autora desta dissertação declara ter sido a principal executante do trabalho experimental, tendo activamente participando na concepção do desenho experimental, na interpretação dos resultados obtidos e na redação dos manuscritos.

Os estudos apresentados nesta dissertação foram realizados no grupo de investigação "Neuron Glia Biology in Health & Disease", Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculdade de Farmácia da Universidade de Lisboa, sob a orientação dos Professores Dora Brites e Adelaide Fernandes. Parte do trabalho foi também realizado no "MRC Centre for Regenerative Medicine Scottish Centre for Regenerative Medicine" Universidade de Edimburgo, Escócia, sob a orientação do Professor Doutor Charles ffrench-Constant e no Instituto de Biologia Molecular e Celular da Universidade do Porto, Portugal, sob a orientação do Professor Doutor João Bettencourt Relvas.

O trabalho foi subsidiado pelos projectos PEst-OE/SAU/UI4013/2011 concedido ao iMed.UL e PTDC/SAU-NEU/64385/2006 concedido à Professora Doutora Dora Brites pela Fundação para a Ciência e Tecnologia (FCT), sendo que a Autora usufruiu de uma bolsa de Doutoramento (SFRH/BD/43885/2008) concedida pela FCT, Lisboa, Portugal.

Para os meus pais

Acknowledgements/Agradecimentos

São muitas as pessoas que estiveram ao meu lado e às quais tenho de agradecer por me terem não só ajudado a alcançar esta meta, mas também a tornar-me na pessoa que hoje sou.

Em primeiro lugar quero naturalmente agradecer à Professora Doutora Dora Brites, orientadora deste trabalho. Agradeço-lhe por me ter aceite no seu grupo ainda como bolseira de investigação, numa fase em que não tinha qualquer tipo de experiência na área das neurociências. O seu elevado padrão de rigor e a constante procura por um trabalho mais completo, fizeram com que eu acreditasse sempre que seria possível chegar ao final desta etapa com um trabalho de elevada qualidade, mesmo quando todos os obstáculos fariam prever que isso não seria possível. Obrigada ainda por toda a confiança que depositou em mim, dando-me a oportunidade de estar em contacto com várias situações com as quais nunca tinha tido oportunidade de me defrontar. Assim, gostaria de lhe agradecer por me ter encorajado sempre a alcançar as pequenas metas ao longo dos anos e por ter contribuído de forma muito positiva para que me tornasse na profissional que me tornei. Foi um prazer trabalhar consigo ao longo destes cinco anos e espero ter conseguido responder a todos os desafios que me colocou com a qualidade que pretendia.

Agradeço também à Professora Doutora Adelaide Fernandes, co-orientadora deste trabalho. Muito poderia ser dito sobre tudo aquilo que tenho para te agradecer... A nível profissional, gostava de te agradecer por tudo aquilo que me ensinaste e por me teres sempre incentivado a querer mais e melhor. Obrigada pela tua disponibilidade em todos os momentos críticos e obstáculos que foram surgindo ao longo desta etapa que nem sempre foi fácil de ser percorrida. Obrigada por acreditares sempre em mim e espero estar sempre à altura das expectativas que depositas em mim. Mas como sabes, para além de orientadora tornaste-te também ao longo destes anos numa das minhas melhores amigas, com quem sei que poderei contar sempre. Tens sido uma das pessoas sempre disponível para me ouvir, para me dar a mão quando preciso dela para me levantar, para me lembrar que mereço mais e melhor e que cair não será nunca uma solução. A tua amizade foi uma das coisas mais importantes a nível pessoal que ganhei ao longo destes últimos anos. Muito obrigada por seres a pessoa que és...

À Professora Doutora Alexandra Brito e ao Professor Doutor Rui Silva, por se terem mostrado sempre disponíveis para me ajudar sempre que precisei dos seus conhecimentos. Muito obrigada por tudo o que me transmitiram e que sem dúvida me ajudaram bastante a crescer a nível profissional.

I would also like to thank Professor Charles ffrench-Constant and his lab for welcoming me in Edinburgh during my training period and for providing me so much valuable knowledge.

Ao Professor Doutor João Relvas, por me ter recebido no seu laboratório no Porto com tanta simpatia. Muito obrigada por se ter mostrado sempre disponível para discutir o trabalho e por me ter transmitido importantes conhecimentos.

Tenho também de agradecer a todas as meninas que fazem parte do grupo a que pertenço e que tanto contribuem para o bom ambiente que se vive nele e que tornam mesmo os dias mais complicados em dias um pouquinho mais coloridos!

E aqui como é claro a minha primeira palavra de agradecimento vai para a Inês... Foste muito mais que uma colega de trabalho, foste até muito mais que uma amiga! Acho que a empatia que criámos vem desde os primeiros dias em que chegaste ao laboratório... Desde aí que a nossa amizade tem vindo a crescer e a tornar-se mais forte... E hoje és uma das minhas melhores amigas, uma pessoa muito especial no meu percurso... Foste sempre a minha terceira mão quando apenas duas não chegavam para as pipetas! E ainda mais importante que isso foste sempre uma amiga disponível para me ouvir, o que nem sempre se tornou fácil ao longo deste último ano! Obrigada por estares sempre lá, por me tentares fazer acreditar que a vida tem cor! Por me fazeres sorrir quando essa é a última coisa que quero fazer, quando apenas as lágrimas querem surgir no meu rosto... Espero ter retribuído a tua amizade e desejo-te toda a sorte do Mundo para completares também esta etapa! Que a nossa amizade continue a fazer-nos sorrir daqui para a frente... Aproveito aqui para fazer também um pequeno parênteses, para agradecer ao Mário (“Eu sou tipo..... Tramendo!) por todo o apoio que também me deu, por ter sempre uma palavra amiga e por ser o meu amigo para ver os jogos do Glorioso! Sempre a marcar pontos! Consegui juntar as palavras tramendo e Glorioso na mesma frase! Obrigada aos dois por me fazerem sentir tão especial...

À Dudu... Foi pouquinho o tempo que passaste connosco em Lisboa, mas o que nós criámos foi tão especial que não quero perder nunca... Foram tantos os momentos que passámos juntas, tantas as risadas, tanta a cumplicidade! És uma amiga muito especial com a qual sei que poderei contar sempre e com quem tu também sempre poderás contar... És uma daquelas pessoas que também é sempre capaz de me arrancar um sorriso e que me faz sentir sempre que também eu sou especial... Gosto tanto de ti... Um beijinho muito especial à minha sobrinha emprestada Beatriz que tantos sorrisos me arrancou...

À Ritinha gostava de agradecer todo o apoio e amizade que demonstrou desde que cheguei e ao grupo! Tu e a Sandrinha foram os meus braços direitos nos primeiros anos e muito do que aprendi devo-vos a vocês! Voltando a ti, muito obrigada por todas

as conversas, por todos os momentos, por todos os lanchinhos que serviam para descontrair nos dias menos fáceis! Ainda bem que voltaste e espero que continuemos a percorrer estes caminhos da ciência juntas!

Sandrinha, tu foste mais uma das pessoas que já não partilhas o dia a dia connosco, mas sem dúvida que foste uma pessoa muito marcante no tempo que passámos juntas... Muito obrigada por toda a disponibilidade e amizade que mostraste ao longo do tempo que passámos juntas! Tanto que nós nos rimos e por isso apesar de já não estares por cá espero que continuemos a partilhar a amizade que temos. Por seres a pessoa que és desejo que tudo te corra pelo melhor.

Quero também agradecer à Sofia, por me ter ajudado sempre e por ser a simpatia de pessoa que é... Sei que vou poder sempre contar contigo! À Filipa, por todos os bons momentos e por todas aquelas frases que soltas do nada, quando nada fará prever, que só tu é que percebes, mas que no final nos arrancam quase sempre uma boa gargalhada! À Belli, a nossa brasileirinha, que se mostrou sempre tão amiga... Com a qual ainda tenho o privilégio de poder continuar a contar e a partilhar momentos, mesmo apesar de estares longe, longe! Espero que o interrupitô da nossa amizade continue sempre ligado!

E por fim mas não por último, às novas meninas de mestrado e Doutoramento! Cátia, apesar de todas as promessas de desagradecimentos não sou capaz de o fazer... És uma pessoa da qual gosto muito, que se tem mostrado sempre amiga, apesar das perguntas constantes do “Então já está quase?”. E pois é está mesmo quase e muito contribuíste para que a minha boa disposição se mantivesse nestes últimos tempos... À Jokinha, que por vezes parece ter um parafuso a menos! ☺ És mais uma daquelas pessoas que todos os dias me arranca uma boa gargalhada! Por isso muito obrigada!!! Obrigada também por me mostrares que posso sempre contar contigo! Quero também agradecer à Cláudia pela disponibilidade e por todas as conversas. E também à Gisela por todos os momentos que temos vindo a partilhar. A todas desejo muito boa sorte na vossa caminhada...

I would also to thank you people from Edinburgh lab. Especially to Veronique Miron, I would like to thank you all the support and knowledge during my stay in Edinburgh. Thank you for all the effort and willingness to allow the achievement of all the proposed objectives during the short period of time that I spent in Edinburgh. Thank you also by your friendship and for all good times that we spent outside the lab. Tenho ainda de fazer um agradecimento muito especial à Cristina, por toda a ajuda que me deu na minha chegada a Edimburgo e ao longo de toda a estadia por esses lados. Muito obrigada por me teres sempre incluído nos teus planos e por me teres mostrado que há

pessoas com as quais podemos contar sempre! A minha estadia em Edimburgo teria sido muito mais cinzenta sem a tua amizade...

A todas as pessoas do IBMC que sempre me ajudaram em mais uma estadia num laboratório desconhecido! ☺ Especialmente, à Doutora Sofia Domingues que tão bem me acolheu no laboratório do Professor Doutor João Relvas. Tenho sem dúvida de te agradecer por toda a ajuda que me deste durante a minha estadia no Porto. A tua disponibilidade tornou-se ainda mais importante, pois nem todas as etapas foram fáceis de alcançar e porque o tempo também não abundava. Muito obrigada por todo o conhecimento que me transmitiste e por teres sempre mostrado interesse em discutir o trabalho comigo, pois esses momentos foram sempre bastante importantes para definir percursos e tornar o trabalho sempre em algo mais completo. Obrigada ainda pela tua amizade e por todos os momentos que passamos juntas. Gostaria também, sem dúvida, de agradecer à Doutora Sofia Santos, por toda a sua simpatia com que me recebeu no mesmo laboratório. Muito obrigada pelos conhecimentos científicos que me transmitiste e que tão úteis se tornaram para melhorar o meu trabalho. Obrigada pela disponibilidade demonstrada sempre que precisei da tua ajuda. Um beijo especial também para a Pipas, para a Joana, para a Débora, para a Ana e para o Nuno por me terem ajudado sempre que precisei e por me terem proporcionado bons momentos no Porto!

A todos colegas das catacumbas queria agradecer todos os bons momentos e toda a simpatia que sempre mostraram ao longo destes anos. Tenho sem dúvida que deixar uma palavra especial ao meu primeiro vizinho, ao Pedro. Porque a vida fez com que nos tornássemos mais que colegas, obrigada pela tua amizade... Muito obrigada pela tua disponibilidade para me ouvires e por todos os conselhos.

A todos os meus amigos e familiares por todo o apoio que me têm dado ao longo de todo o meu crescimento como pessoa. Tenho de destacar todas as meninas do hóquei, que sempre tiveram muita paciência para me ouvir a falar sobre *oligodendrocytes*!!! E mesmo agora que já não nos aturamos todos os dias sei que posso sempre contar com todas vocês! À Leonor que mesmo longe continua a ser uma pessoa muito especial com a qual posso contar sempre para me ajudar e para me fazer sorrir! E à Lu que foi desde sempre uma grande amiga! Mais recentemente, à Gi e à Ana por me ajudarem sempre que preciso!

À Belinha e ao Cidade... Vocês são sem dúvida amigos muito especiais... Espero poder continuar a contar sempre com vocês porque gosto mesmo muito de vocês! Podem continuar a contar comigo sempre...

À Sara e ao Tiago muito obrigada por me terem acolhido tão bem no Porto! Adorei conhecer-vos e não tarda estou aí para uma visita!!! Tiago, és sem dúvida uma pessoa que posso dizer que tenho muito privilégio em conhecer, sempre pronto para me

ajudar, para me ouvir e para ter uma palavra amiga sempre que precisei... Fico muito contente que a minha amiga te tenha encontrado!!!

À Claudinha... Não tenho palavras para descrever tudo o que sinto quando penso o quanto te tenho a agradecer... És sem dúvida nenhuma uma das minhas melhores amigas... Aquela com quem sei que posso contar sempre... Principalmente quando atendes o telemóvel! Brincadeirinha!!! Mesmo longe és a pessoa que está sempre pronta para estar ao meu lado! E se algumas dúvidas eu tivesse disso, ficaria sem nenhumas neste último ano... Obrigada por seres a pessoa extraordinária que neste último ano, que foi tão difícil, esteve sempre ao meu lado... Obrigada pela tua palavra amiga, por me mostrares que valho a pena, mesmo quando a única coisa que penso é que não é assim... Obrigada por me dares sempre a mão, por me limpares as lágrimas, por rires comigo, por me mostrares que haverá sempre um caminho para ser feliz... Adoro-te...

Ao Pedro... Apesar deste último ano ter sido um ano de desencontros que muito me fizeram sofrer... Não posso deixar de te agradecer por tudo aquilo que passámos juntos... Obrigada por me tentares sempre fazer acreditar que seria capaz de chegar ao fim desta etapa... A vida sem ti não teria sido a mesma coisa com toda a certeza! Apesar de tudo espero que as nossas vidas se voltem a encontrar pois és uma pessoa muito especial para mim... Gosto muito de ti...

Aos meus avós, muito obrigada por estarem sempre presentes na minha vida, por me fazerem crescer da melhor forma e por mostrarem todos os dias que me amam acima de tudo... Espero continuar a poder contar com o vosso amor e com a vossa presença por muitos anos...

E por fim como não poderia deixar de ser um obrigada do tamanho do Mundo aos meus pais!!! Porque a vocês vos devo tudo... A pessoa que sou, tudo o que me deram, o amor incondicional que me dão todos os dias... Sem vocês nada teria sido possível!!! Não existem palavras para demonstrar tudo aquilo que sinto por vocês e para expressar toda a gratidão que sinto!!! Muito obrigada por me fazerem acreditar todos os dias que é possível e que eu vou ser capaz de atingir tudo aquilo que me proponho! Por isso queria partilhar esta tese com vocês! Ela também é um bocadinho vossa... Amo-vos muito…

Index

ABBREVIATIONS ..... **xxxi**

ABSTRACT ..... **xxxv**

RESUMO ..... **xxxvii**

CHAPTER I - GENERAL INTRODUCTION **1**

1. BRAIN NETWORK: THE NEURON-GLIA INTERPLAY **3**

1.1. Neurons 4

1.2. Astrocytes 5

1.3. Microglia 6

1.4. Oligodendrocytes 8

2. OLIGODENDROCYTES DURING CENTRAL NERVOUS SYSTEM DEVELOPMENT **9**

2.1. Oligodendrocyte origin 9

2.2. Temporal oligodendrocyte lineage progression 10

2.3. Myelination 13

2.4. Regulation of oligodendrocyte development and myelination 14

2.4.1. Transcription factors 15

2.4.2. Cytoskeleton components and their regulation 15

2.4.3. Extracellular factors 17

2.4.3.1. Growth factors 17

2.4.3.2. Cytokines and chemokines 18

2.4.4. Neuron-oligodendrocyte communication 19

2.4.4.1. Neurotransmitters and their receptors 19

2.4.4.2 Cell adhesion molecules 20

3. MECHANISMS OF OLIGODENDROCYTE INJURY **22**

3.1. Molecular mechanisms 23

3.1.1. Excitotoxicity 23

3.1.2. Oxidative damage 24

3.1.3. Endoplasmic reticulum stress 25

3.1.4. Cytokines 28

3.2. Mechanisms mediated by other central nervous system cells 29

4. MODELS TO ASSESS CHANGES IN OLIGODENDROCYTE DEVELOPMENT AND MYELINATION **30**

5. OLIGODENDROCYTE INVOLVEMENT IN SOME NEUROLOGICAL DISORDERS **33**

5.1. Schizophrenia 33

5.2. Multiple sclerosis 35

5.3. Neonatal hyperbilirubinemia 37

5.3.1. Overview to the mechanisms underlying bilirubin-induced toxicity 40

5.3.2. Does bilirubin compromise myelination? 42

6. GLOBAL AIMS OF THE THESIS 46

7. REFERENCES 48

CHAPTER II - ER STRESS, MITOCHONDRIAL DYSFUNCTION AND CALPAIN/JNK ACTIVATION COOPERATE TO TRIGGER OLIGODENDROCYTE PRECURSOR CELL DEATH BY UNCONJUGATED BILIRUBIN 81

ABSTRACT 83

1. INTRODUCTION 84

2. MATERIAL AND METHODS 86

2.1. Animals 86

2.2. Primary culture of OPC 87

2.3. OPC treatment 87

2.4. Cell death assays 88

2.5. Caspase activity determination 89

2.6. Assessment of mitochondrial dysfunction 89

2.7. Measurement of ROS production 90

2.8. Measurment of calpain activation 90

2.9. Western blot assay 91

2.10. Densitometry and statistical analysis 91

3. RESULTS 91

3.1. Phenotypic characterization of enriched populations of OPC 91

3.2. UCB causes a decrease in OPC viability 93

3.3. UCB increases the production of ROS by OPC, but not that of cytokines or glutamate 97

3.4. UCB triggers mitochondrial dysfunction and caspase-9 cascade activation in OPC 98

3.5. UCB activates ER stress pathway in OPC 99

3.6. UCB triggers the activation of JNK signalling pathway in OPC 101

3.7. UCB upregulates calpain activity in OPC 102

4. DISCUSSION 104

5. REFERENCES 109

6. SUPPLEMENTARY FIGURES 116

6.1. Supplementary Figure 1 116

6.2. Supplementary Figure 2 117

CHAPTER III - ARREST OF OLIGODENDROCYTE DIFFERENTIATION AND AXONAL MYELINATION IN AN *IN VITRO* MODEL OF HYPERBILIRUBINEMIA 119

ABSTRACT 121

1. INTRODUCTION 122

2. MATERIAL AND METHODS 124

2.1. Reagents and media 124

2.2. Animals 125

2.3. Oligodendrocyte cell culture and treatment 125

2.4. Myelinating co-culture and treatment 126

2.5. Immunocytochemistry and data analysis of primary oligodendrocyte cell culture 126

2.6. Quantitative PCR 127

2.7. GTPase Pull-Down Assays 128

2.8. Immunocytochemistry and data analysis of myelinating co-culture 128

2.9. Statistical analysis 129

3. RESULTS 130

3.1. UCB impairs the differentiation of oligodendrocyte progenitors into myelinating cells 130

3.2. UCB prevents oligodendrocyte processes extension, myelin membrane formation and impairs Rac activation 132

3.3. UCB impairs differentiation of oligodendrocyte progenitors into myelinating cells in myelinating co-cultures 134

3.4. UCB reduces oligodendrocyte process extension towards the axon and myelination 135

4. DISCUSSION 137

5. REFERENCES 141

6. SUPPLEMENTARY FIGURES 146

6.1. Supplementary Figure 1 146

CHAPTER IV - CEREBELLUM SLICE CULTURE MODEL MIMICKING HYPERBILIRUBINEMIA: CHANGES IN OLIGODENDROCYTE MATURATION, MYELINOGENESIS AND GLIAL REACTIVITY 147

ABSTRACT 149

1. INTRODUCTION 150

2. MATERIAL AND METHODS 152

2.1. Reagents and media 152

2.2. Animals 152
2.3. Cerebellar slice culture and treatment 153
2.4. Immunostaining procedure 154
2.5. Semi-quantitative RT-PCR 154
2.6. Cytokine determination 155
2.7. S100B determination 155
2.8. Glutamate determination 156
2.9. Statistical analysis 156

3. RESULTS 156
3.1. UCB treatment impairs oligodendrocyte maturation and myelination 156
3.2. Differences in Olig1 and Olig2 mRNA expression are involved in oligodendrocyte maturation impairment by UCB 158
3.3. UCB treatment stimulates astrogliosis and microgliosis 159
3.4. UCB treatment induces the secretion of TNF-α while inhibits the release of OL-6 161
3.5. Treatment of cerebellar slice cultures with UCB leads to a fast and transient release of glutamate 162

4. DISCUSSION 163

5. REFERENCES 167

CHAPTER V - REDUCED MYELINATION AND INCREASED GLIA REACTIVITY IN A HYPERBILIRUBINEMIC MOUSE MODEL 175

ABSTRACT 177

1. INTRODUCTION 178

2. MATERIAL AND METHODS 180
2.1. Animals 180
2.2. Immunohistochemistry 180
2.3. Measuring myelination and counting Iba-1 and GFAP-labelled cells 181
2.4. Statistical analysis 182

3. RESULTS 182
3.1. Alterations in brain morphology of kernicterus mice 182
3.2. Kernicterus mice present axonal loss and decreased myelination 183
3.3. Kernicterus mice present reduced myelination and increased glial reactivity in cerebellum, medulla oblongata and pons 184
3.4. Distinct white matter regions from cerebellum present different alterations in myelination and glial reactivity 187

3.5. Kernicterus mice present cerebellum atrophy and a great reduction in Purkinje cell number 190

4. DISCUSSION 191

5. REFERENCES 194

CHAPTER VI - FINAL CONSIDERATIONS 199

1. CONCLUDING REMARKS AND PERSPECTIVES 201

2. REFERENCES 208

Figure index

CHAPTER I - GENERAL INTRODUCTION 1

Fig. I.1 – Brain network organization 3

Fig. I.2 – Four steps of oligodendrocyte maturation towards oligodendroglial lineage: oligodendrocyte progenitor cells (OPC), preoligodendrocytes, immature oligodendrocytes and mature oligodendrocytes 11

Fig. I.3 – Comparison between oligodendrocyte lineage development in human and rodents .. 13

Fig. I.4 – Regulation of the actin cytoskeleton by Rho GTPases 17

Fig. I.5 – Oligodendrocyte development and myelination are regulated by neuronal signals. ... 22

Fig. I.6 – Mechanisms of toxicity to oligodendrocytes. 25

Fig. I.7 – Signal transduction events associated with endoplasmic reticulum (ER) stress 27

Fig. I.8 – Models to evaluate oligodendrocyte (OL) development and myelination 32

Fig. I.9 – Major findings regarding the molecular mechanisms on cell function induced by unconjugated bilirubin (UCB) 42

Fig. I.10 – Bilirubin subcellular localization in the rat brain 10 min after an I.V. administration of a bilirubin bolus of 50 mg/kg over a period of 5 min 43

Fig. I.11 – Ultrastructural abnormalities observed in asphyxiated rabbits with less than 24 hours old after an intravenous injection of bilirubin (a) and in the hyperbilirubinemic Gunn rat model (aged 4 weeks) (b). 44

CHAPTER II - ER STRESS, MITOCHONDRIAL DYSFUNCTION AND CALPAIN/JNK ACTIVATION COOPERATE TO TRIGGER OLIGODENDROCYTE PRECURSOR CELL DEATH BY UNCONJUGATED BILIRUBIN 81

Fig. II.1 – Phenotypic characterization of the enriched culture of oligodendrocyte precursor cells obtained from neonatal rats 92

Fig. II.2 – Unconjugated bilirubin (UCB) induces cell death by apoptosis in oligodendrocyte precursor cells (OPC) 94

Fig. II.3 – Unconjugated bilirubin (UCB) induces necrosis-like oligodendrocyte precursor cell (OPC) death 95

Fig. II.4 – Generation of reactive oxygen species (ROS) by oligodendrocyte precursor cells (OPC) is enhanced upon unconjugated bilirubin (UCB) stimulation 97

Fig. II.5 – Unconjugated bilirubin (UCB) decreases mitochondrial viability and activates caspase cascade in oligodendrocyte precursor cells (OPC)..... 99

Fig. II.6 – Unconjugated bilirubin (UCB) induces endoplasmic reticulum stress in oligodendrocyte precursor cells (OPC)..... 101

Fig. II.7 – Unconjugated bilirubin (UCB) stimulates the activation of JNK pathway in oligodendrocyte precursor cells (OPC)..... 102

Fig. II.8 – Calpain activation in oligodendrocyte precursor cells (OPC) increases upon exposure to unconjugated bilirubin (UCB)..... 103

Fig. II.9 – Schematic representation of the potential intracellular pathways and subcellular compartments involved in unconjugated bilirubin (UCB) injury to oligodendrocyte precursor cells (OPC) 108

Suppl. Fig. II.1 – Inhibition of caspases, calpains and oxidative stress prevented unconjugated bilirubin (UCB)-induced oligodendrocyte precursor cell (OPC) death 116

Suppl. Fig. II.2 – Unconjugated bilirubin (UCB) induces an increase in activating transcription factor 6 (ATF-6) and inositol-requiring enzyme 1α (IRE-1α), chaperones usually involved in endoplasmic reticulum stress, in oligodendrocyte precursor cells (OPC)..... 117

CHAPTER III - ARREST OF OLIGODENDROCYTE DIFFERENTIATION AND AXONAL MYELINATION IN AN *IN VITRO* MODEL OF HYPERBILIRUBINEMIA 119

Fig. III.1 – Unconjugated bilirubin (UCB) impairs oligodendrocyte maturation if exposure occurs during the proliferation stage 131

Fig. III.2 – Oligodendrocyte morphological maturation and mature oligodendrocyte diameter are modified by unconjugated bilirubin (UCB) treatment 132

Fig. III.3 – Unconjugated bilirubin (UCB) reduces the primary branching density and elongation of mature oligodendrocytes 133

Fig. III.4 – Unconjugated bilirubin (UCB) reduces Rac1 activity..... 134

Fig. III.5 – Unconjugated bilirubin (UCB) impairs oligodendrocyte maturation in a co-culture myelinating model if exposure occurs before the beginning of myelination..... 135

Fig. III.6 – In a myelinating co-culture model, unconjugated bilirubin (UCB) is able to induce a decrease in oligodendrocyte morphological maturation 136

Fig. III.7 – Unconjugated bilirubin (UCB) treatment leads to a decrease in the number of internodes myelinated per each oligodendrocyte, as well as in the internode length..... 137

Suppl. Fig. III.1 – Unconjugated bilirubin (UCB) did not induce axonal damage 146

CHAPTER IV - CEREBELLUM SLICE CULTURE MODEL MIMICKING HYPERBILIRUBINEMIA: CHANGES IN OLIGODENDROCYTE MATURATION, MYELINOGENESIS AND GLIAL REACTIVITY 147

Fig. IV.1 – Impairment of oligodendrocyte (OL) differentiation by unconjugated bilirubin (UCB), in a rat cerebellar slice culture, increase with the time of exposure 157

Fig. IV.2 – Impaired myelination by unconjugated bilirubin (UCB) in rat cerebellar slice cultures increases with the time of exposure 158

Fig. IV.3 – Unconjugated bilirubin (UCB) induces a reduction and an increase of Olig1 and Olig2 mRNA expression, respectively 159

Fig. IV.4 – Unconjugated bilirubin (UCB) induces an increase in the number of microglia and astrocytes 160

Fig. IV.5 – Unconjugated bilirubin (UCB) leads to an increase in TNF-α secretion and a decrease in that of IL-6 162

Fig. IV.6 – Unconjugated bilirubin (UCB) induces glutamate release immediately after the treatment period 163

CHAPTER V - REDUCED MYELINATION AND INCREASED GLIA REACTIVITY IN A HYPERBILIRUBINEMIC MOUSE MODEL 175

Fig. V.1 – *Kernicterus* mice present axonal loss and decreased myelination 183

Fig. V.2 – Reduced myelination and increased glial reactivity were observed in *kernicterus* mice, in specific regions, such as cerebellum, medulla oblongata and pons 185

Fig. V.3 – Reduced myelinated fibers and increased microglia and astroglia burden in the *kernicterus* mice, in specific regions, such as cerebellum, medulla oblongata and pons 186

Fig. V.4 – Myelination and glial reactivity changes in different areas of cerebellum white matter in the *kernicterus* mice 189

Fig. V.5 – Reduced myelinated fibers and increased microglia and astroglia burden in the *kernicterus* mice, different changes throughout white matter areas 189

Fig. V.6 – *Kernicterus* mice present cerebellum atrophy, loss of Purkinje cells and less branching in the remaining cells 190

CHAPTER VI - FINAL CONSIDERATIONS..... 175

Fig. VI.1 – Schematic representation of the major findings achieved in the present work 207

CHAPTER II - ER STRESS, MITOCHONDRIAL DYSFUNCTION AND CALPAIN/JNK ACTIVATION COOPERATE TO TRIGGER OLIGODENDROCYTE PRECURSOR CELL DEATH BY UNCONJUGATED BILIRUBIN 81

Table II.1 – Temporal changes in oligodendrocyte viability by incubation with unconjugated bilirubin 96

Table II.2 – Oligodendrocyte precursor cell death by unconjugated bilirubin is partially prevented by Z-VAD-FMK, N-acetylcysteine (NAC) and calpeptin 96

Abbreviations

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
Arp2/3	Actin-related protein 2/3
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BIND	Bilirubin-induced neurological dysfunction
BIP	Immunoglobulin-heavy-chain-binding protein
CD	Cluster of differentiation
CHOP	CCAAT/enhancer-binding protein homologous protein
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous sytem
CNTF	Ciliary neurotrophic factor
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
E	Embryonic day
EAAT	Excitatory amino acid transporter
EGF	Epidermal growth factor
eIF2α	Eukariotic translation-initiation factor 2α
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
FGF-2	Fibroblast growth factor 2
GABA	γ-aminobutyric acid

GADD34	Growth-arrest DNA damage gene 34
GalC	Galactocerebroside C
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GRP	Glucose-regulated protein
GTP	Guanosine triphosphate
HBMEC	Human brain microvascular endothelial cells
Iba	Ionized calcium binding adaptor molecule
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IRE-1α	Inositol-requiring enzyme 1α
JNK	c-Jun *N*-terminal kinase
LIF	Leukemia inhibitory factor
LINGO-1	Leucine-rich repeat- and Ig domain-containing nogo receptor-interacting protein 1
MAG	Myelin associated glycoprotein
MBP	Myelin basic proteins
MF	Microfilaments
MAP	Microtubule associated protein
MAPK	Mitogen-protein kinase
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
Mrp1	Multidrug resistance-associated protein 1

MS	Multiple sclerosis
MT	Microtubules
NF-κB	Nuclear factor-kappaB
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NT-3	Neurotrophin 3
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
P	Post-natal day
PARP	Poly(adenosine diphosphate-ribose) polymerase
PDGF-A	Platelet derived growth factor A
PDGF-Rα	Platelet-derive growth factor receptor α
PERK	Pancreatic endoplasmic reticulum kinase
Pgp	P-glycoprotein
PLP	Proteolipid protein
pMN	Motor neuron precursors
PPMS	Primary progressive multiple sclerosis
PSA-NCAM	Polysialic acid-neural cell adhesion molecule
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
SC	Spinal cord

Shh	Sonic hedgehog homolog
SPMS	Secondary progressive multiple sclerosis
T3	Thyroid hormone
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRAF2	TNF-α-receptor-associated factor 2
TSB	Total serum bilirubin
UCB	Unconjugated bilirubin
UGT	Uridine 5'-diphospho-glucuronosyl transferase
UPR	Unfolded protein response
WASP	Wiskott-Aldrich syndrome protein
WAVE	Wiskott-Aldrich syndrome protein family verprolin homologs
XBP1	X-box binding protein 1
XBP1s	Spliced XBP1

Abstract

Oligodendrocytes (OL) are neuroglial cells present in the central nervous system responsible for myelin sheath formation, that provide an electric insulation of axons fastening the transmission of electrical signals. During the neonatal period, the brain is particularly vulnerable to toxic insults, like the one induced by hyperbilirubinemia. It has been demonstrated that in this condition, high levels of unconjugated bilirubin (UCB) are toxic to endothelial cells, neurons, astrocytes and microglia. However, the result of oligodendrocyte precursor cell (OPC) exposure to UCB has never been explored. Thus, this thesis intended to access the vulnerability of OPC to UCB, in conditions mimicking neonatal hyperbilirubinemia, and dissect the mechanisms that might be impared through OPC maturation and neuronal myelination.

First, we observed that UCB induced OPC apoptosis and necrosis-like cell death. This increase revealed to be due to early signals of endoplasmic-reticulum (ER) stress, followed by c-Jun *N*-terminal kinase 1/2 activation, mitochondrial dysfunction and reactive oxygen species (ROS) production. Resulting changes in intracellular calcium homeostasis, led to subsequent calpain activation. In addition, when specific inhibitors of caspases, calpains and oxidative stress were used, we observed prevention in UCB-induced OPC death. We next verified, by using an *in vitro* OL maturation model, that UCB impaired OL differentiation, increasing the number of immature cells, involving changes in the messenger ribonucleic acid expression of Olig1 and Olig2 transcription factors. Moreover, UCB led to changes in the morphology of mature cells, decreasing the diameter and the number of primary processes, as a result of the decreased proportion of active Rac1. Using a myelinating co-culture model we were able to show that UCB induced not only similar effects in OL differentiation and maturation, but also a decrease in the myelination pattern, with consequent decrease both in the number of myelinated internodes and in internode length. These myelination-related alterations were corroborated in organotypic cerebellar slice cultures, where we also observed microgliosis and astrogliosis, increased inflammation and glutamate release upon exposure to UCB. Finally, in an animal model of severe hyperbilirubinemia we found myelin deficits and changes in astrocyte and microglia number in several brain regions, including cerebellum, medulla oblongata and pons. Interestingly, cerebellum evidenced the highest reduction in volume, together with a marked impairment in Purkinje cells.

In conclusion, our findings suggest that UCB compromises oligodendrogenesis and OL ability to myelinate, thus compromising axonal function and transmission of the nerve impulse. This intervention in neurodevelopment impairment

in jaundiced babies may additionally turn out an increased vulnerability to aging and/or appearance of neurological diseases.

Keywords: Astrocytes; Hyperbilirubinemia; Intercellular communication; Intracellular signalling; Microglia; Myelination; Neuroinflammation; Neurons; Oligodendrocytes.

Resumo

Os oligodendrócitos (OL) são células gliais existentes no sistema nervoso central dos vertebrados. Estas células são responsáveis pela formação de uma das estruturas mais especializadas nos humanos, as bainhas de mielina. Estas fornecem não apenas suporte aos axónios, mas também permitem a propagação saltatória dos impulsos nervosos nos neurónios, tornando assim bastante mais rápida a transferência de informação entre as diferentes regiões cerebrais.

A progressão destas células ao longo da sua maturação envolve vários passos de diferenciação que são identificáveis de acordo com um aumento da sua complexidade morfológica, com uma diminuição da sua capacidade migratória e com o padrão de expressão de marcadores específicos. Assim, durante a maturação, os OL vão adquirindo uma morfologia cada vez mais complexa e perdem a sua capacidade migratória e proliferativa. Inicialmente os OL apresentam uma morfologia bipolar e possuem uma elevada capacidade proliferativa, sendo denominados como oligodendrócitos precursores (OPC). Posteriormente os OPC dão origem a OL mais diferenciados que apresentam uma morfologia mais complexa composta por vários processos. Nos estádios finais do seu desenvolvimento, os OL maduros são células que expressam proteínas da mielina, como a proteína básica da mielina (MBP), e que já estão em contacto com os axónios de modo a envolvê-los com as bainhas de mielina.

Possívelmente devido ao seu complexo programa de diferenciação e ao seu elevado metabolismo, os OL são considerados como o tipo de células mais vulneráveis do sistema nervoso central. Para além disso, perturbações na mielinização durante o período neonatal poderão originar disfunção axonal e défices na condução dos impulsos nervosos, levando a alterações na comunicação cerebral com possíveis sequelas neurológicas a longo termo.

A hiperbilirrubinémia é uma condição muito frequente durante o período neonatal que resulta de níveis elevados de bilirrubina não conjugada (BNC) em circulação. A BNC é formada pelo catabolismo do heme, sendo que nos recém-nascidos cerca de 75% da sua totalidade é proveniente do catabolismo da hemoglobina. Durante o período neonatal, dada a fisiologia do recém-nascido, ocorrem diversas alterações no metabolismo da bilirrubina que levam a um aumento dos seus níveis em circulação. As consequências adversas da elevação dos níveis de BNC em circulação são várias podendo causar lesões reversíveis, que se podem tornar em irreversíveis em situações extremas de hiperbilirrubinémia, denominadas por *kernicterus*, nas quais se verifica deposição de BNC em determinadas regiões do sistema nervoso central.

Estudos prévios conduzidos no nosso laboratório demonstraram que níveis elevados de bilirrubina, mimetizando uma hiperbilirrubinémia moderada, são suficientes para causar toxicidade *in vitro* em neurónios, astrócitos e microglia. Em neurónios observámos que a exposição à BNC leva a um dano oxidativo, libertação de glutamato e alterações na arborização neurítica, sendo as células imaturas as mais susceptíveis ao dano induzido pela BNC. Esta maior vulnerabilidade das células "jovens" foi igualmente observada nos astrócitos. Curiosamente, a BNC é capaz de estimular astrócitos e microglia, levando-as a secretar citocinas pro-inflamatórias como o factor de necrose tumoral (TNF)-α e a interleucina (IL)-1β. Em relação ao efeito da BNC nos OL, existe apenas um estudo que demonstra que os níveis elevados de BNC são responsáveis por um decréscimo na viabilidade dos OL maduros para o qual contribui a produção de óxido nítrico. Estudos mais antigos revelaram que a BNC liga-se à MBP, sendo que foi recentemente demonstrada uma alteração da mielinização num caso de *kernicterus*, com perda de fibras mielinizadas na região do cerebelo. No entanto, tanto quanto sabemos, não foi até hoje avaliado o papel da BNC nos diferentes estádios de desenvolvimento dos OL, bem como na sua maturação e mielinização.

Assim, numa primeira fase deste trabalho (Capítulo II) pretendeu-se avaliar o efeito da UCB na viabilidade dos OPC e elucidar as vias de sinalização envolvidas no processo de morte. Os nossos resultados demonstram que a BNC induz um aumento na morte celular dos OPC por apoptose e necrose. Neste processo de morte celular encontram-se envolvidos, numa fase inicial, sinais de stresse do retículo endoplasmático (ER), havendo uma sobrexpressão dos chaperones: proteína 78 regulada pela glucose (GRP78), enzima dependente do inositol 1α (IRE-1α) e factor activador da transcrição 6 (ATF-6). A alteração no ER leva por sua vez à activação da caspase-2 e das c-Jun *N*-terminal cinase 1 e 2 (JNK 1/2), juntamente com disfunção mitocondrial, caracterizada por perda de potencial da membrana e subsequente activação da caspase-9. Posteriormente observa-se uma activação das calpaínas, o que é indicador de um aumento da concentração intracelular de cálcio devido a uma desregulação da mitocôndria e do ER. A produção de espécies reactivas de oxigénio que encontrámos poderá então resultar do dano mitocondrial e dos seus efeitos secundários, que poderão estar na origem do segundo ciclo de activação de GRP78, IRE-1α, caspase-2 and JNK 1/2. Para que tivéssemos a certeza de que estas vias se encontravam envolvidas na morte dos OPC pela BNC recorreu-se à utilização de inibidores específicos das caspases, das calpaínas e do stresse oxidativo, que confirmaram ser então eficientes na prevenção da morte dos OPC pela BNC.

Numa segunda fase do trabalho (Capítulo III), propusémo-nos avaliar os efeitos da BNC na maturação dos OL e na mielinização. Em relação à maturação dos OL, utilizaram-se culturas primárias de OPC onde se induziu a sua diferenciação. Verificou-se que a exposição à BNC antes do início da diferenciação leva a uma inibição da maturação destas células, resultando assim num número mais elevado de OPC e mais reduzido de OL maduros, quando comparado com células não tratadas. Para este resultado poderão contribuir certas alterações a nível dos factores de transcrição importantes para o desenvolvimento dos OL, dado que observámos uma subexpressão de Olig1 e uma sobrexpressão de Olig2, relativamente aos níveis do ácido ribonucleico mensageiro. Por outro lado, a exposição à BNC antes ou durante a diferenciação, evidenciou induzir alterações na maturação morfológica dos OL, onde as células tratadas apresentavam um diâmetro menor e uma redução do número e extensão dos processos. Foi-se então avaliar o papel da BNC na actividade de GTPases que se sabe regularem a polimerização do citoesqueleto. Neste caso, observou-se um decréscimo da fracção activa da Rac1 após os dois tipos de tratamento. Estes resultados evidenciaram que a BNC não só afecta a capacidade dos OL se diferenciarem, mas também a maturação morfológica necessária à mielinização. Posteriormente, num modelo de mielinização em co-cultura composto por OL e neurónios da raiz do gânglio dorsal, demonstrou-se que o tratamento com BNC antes do início da mielinização leva igualmente a um decréscimo na diferenciação dos OL, com consequente aumento do número de OPC. Curiosamente, ambos os tratamentos, tanto antes, como durante a mielinização, resultaram numa redução de OL mielinizantes, do número de internodos mielinizados por um único OL e do comprimento dos internodos. Deste modo se comprova que a acção nefasta da BNC, se exerce quer na diferenciação dos OL, quer na sua maturação e capacidade para mielinizar.

Numa fase subsequente do trabalho (Capítulo IV), fomos estudar a influência que a presença de células gliais pode ter na resposta dos OL à BNC. Para tal, usou-se um modelo de cultura organotípica do cerebelo que tem a vantagem de manter a cito-arquitectura do tecido e possibilitar as interacções entre células. Os resultados obtidos confirmaram mais uma vez que a BNC causa uma elevação do número de células mais imaturas, os OPC, em paralelo com uma diminuição da percentagem de fibras axonais mielinizadas. Entre as alterações causadas está igualmente o aumento da área ocupada pelos astrócitos e microglia nas regiões que se encontram na proximidade dos circuitos de matéria branca. Confirmando dados anteriores, relativamente aos efeitos imunoestimuladores da BNC, observámos também um aumento significativo da libertação de TNF-α e de glutamato e um decréscimo da

libertação de IL-6, não se observando nenhuma alteração na libertação de IL-1β e de S100B. De salientar, contudo, que todos os efeitos observados mostraram depender do tempo de exposição à BNC, sendo as alterações mais pronunciadas sempre que usámos tratamentos mais prolongados.

Aproveitando a oportunidade de podermos estudar os efeitos de uma hiperbilirrubinémia num murganho que desenvolve *kernicterus* nos primeiros dias de vida, cuja caracterização será brevemente publicada, decidiu-se proceder à avaliação dos efeitos produzidos na mielinização e na proporção de glia (Capítulo V). Os animais, estudados no momento do aparecimento dos sintomas de encefalopatia bilirrubínica, exibiam um decréscimo na proporção de axónios mielinizados, bem como um aumento no número de microglia e de astrócitos no cerebelo, no bulbo raquidiano e na ponte. De todas estas regiões, foi o cerebelo a que sofreu mais alterações, apresentando uma redução do volume relativamente à área cerebral e uma maior alteração no número e na morfologia das células de Purkinje.

Em conclusão, os nossos estudos sugerem que a BNC compromete a oligodendrogénese e a capacidade dos OL de mielinizar, o que poderá comprometer a função axonal e a transmissão de impulsos nervosos, podendo desta forma contribuir para o dano neurológico observado em situações de hiperbilirrubinémia neonatal. Estes resultados têm implicações importantes para o esclarecimento dos mecanismos de neurotoxicidade da BNC e dos seus efeitos nefastos a longo prazo, ao mesmo tempo que propiciam o desenvolvimento de estratégias terapêuticas inovadoras na prevenção da lesão neurológica pela BNC.

Palavras chave: Astrócitos; Comunicação intercelular; Hiperbilirrubinémia; Neuro-inflamação; Sinalização intracelular; Microglia; Mielinização; Neurónios; Oligodendrócitos.

Chapter I

GENERAL INTRODUCTION

1. Brain network: The neuron-glia interplay

The brain is one of the most complex structures of the vertebrates. Part of this complexity is due to the enormous variety of cell types in the brain and interactions that take place within it. Besides neurons, the functioning unit of the central nervous system (CNS), there are two major classes of glial cells: 1) the macroglial cells that include ependymoglia, oligodendroglia and astroglia and 2) the microglia (Kettenmann 2005) (Fig. I.1). Development of the majority of cells of vertebrate CNS is achieved through a common pool of precursor cells that sequentially generate neurons and glial cells (Bayer and Altman 1991). Despite the controversies on the origin of microglia, it is known that these cells are descendants from the mesodermal fetal macrophages that derive from primitive macrophages of the yolk sac (Alliot et al. 1999). In the human CNS, glial cells occupy about half of its volume and out-number neurons, achieving a proportion of around 90% (Allen and Barres 2009; Jessen 2004). During several years, many authors described these glial cells like ”glue” in the brain, only with a passive and supporting role. However, more recently, there is general consensus that glial cells have many other functions that are crucial for the formation, operation and adaptation of neural circuitry, with an active role in brain function and information processing during development and in adulthood (Allen and Barres 2009; Araque and Navarrete 2010).

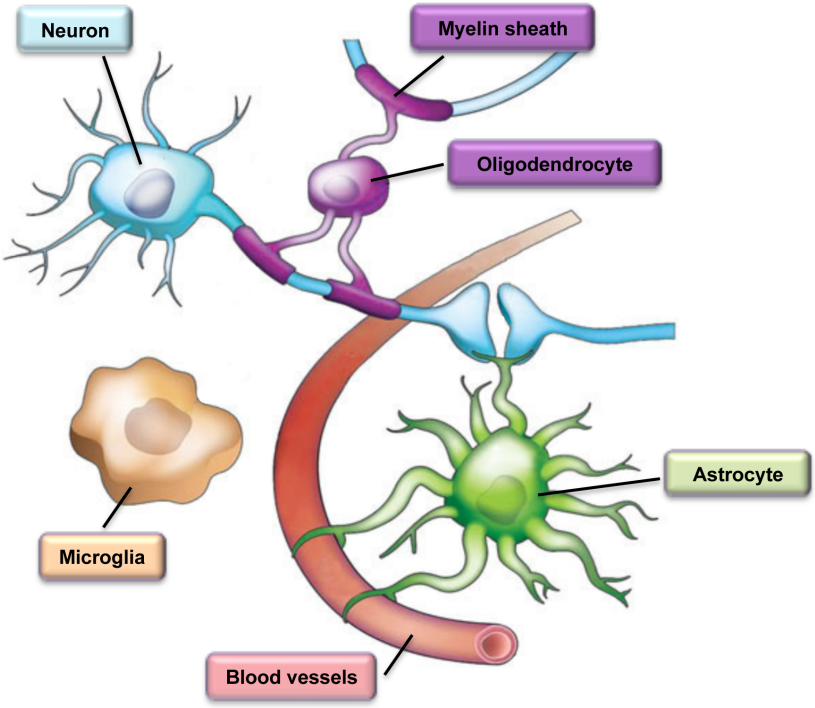


Fig. I.1 – Brain network organization. Glial cells (astrocytes, microglia and oligodendrocytes) establish close connection with neurons and capillary vessels. Oligodendrocytes surround the axons and wrap them, forming the myelin sheath. Adapted from Allen and Barres 2009.

While only neurons are able to carry electrochemical impulses between themselves, glial cells perform many other important functions, including digestion of parts of dead neurons, insulation of axons to speed up electrical communication, as well as physical and nutritional support of neurons (Parpura et al. 2012). The signals between neurons and glia include cell adhesion molecules, ion effluxes, and signalling molecules released from synaptic regions of the neuron, such as neurotransmitters including glutamate or others including fractalkine and nitric oxide (NO) (Liu et al. 2006; Verge et al. 2004). In other hand, glia can affect neuronal excitability and synaptic transmission by releasing signalling molecules that coordinate activity across neuronal networks (Fields and Stevens-Graham 2002). So, glia are very important for brain network formation and maintenance, but if too much activated in response to an insult they are known to contribute either to the development of various diseases, such as neurodevelopmental and neurodegenerative disorders, or to their exacerbation (Liu et al. 2011).

1.1. Neurons

Neurons, the functioning unit of the CNS, develop a highly polarized structure that allows the formation of neural circuits. Although the morphology of various types of neurons differs in some aspects, they all contain four distinct regions: cell body, dendrites, axon and axon terminals. Dendrites are multiple filaments that arise from the cell body and branch multiple times, whereas axons are single and also suffer multiple ramifications. Through these dendrites, neurons receive and integrate information from a vast number of synaptic inputs. The major structural components of dendrites are actin filaments and microtubules that are stabilized by microtubule associated proteins (MAPs), e.g. MAP-2 (Chen and Ghosh 2005). On the other hand, axons are specialized for the conduction of a particular type of electric impulse, designated as action potential, away from the cell body and towards the axon terminus where synaptic contacts are made. Axons are mainly composed by filamentous actin, F-actin, and microtubules that are stabilized by Tau1, another MAP. In humans, the transmission of signals occurs faster and in a saltatory manner, which depends both on the insulating properties of myelin produced by oligodendrocytes (OL) and on the clustering of voltage gated sodium (Na^+) channels at the nodes of Ranvier between OL myelin sheaths (Bradl and Lassmann 2010). In the absence of myelin, the conduction velocity is directly proportional to the diameter of the axon. Synapses, involving the pre-synaptic partner form the axonal terminal plus the post-synaptic component of the target dendrite, are actively formed and remodeled during the first two weeks of

postnatal development, a period of remarkable plasticity. Initially, neurons make many more synaptic connections than those found in mature brain. In fact, along CNS maturation takes place an activity-dependent developmental process termed synaptic pruning, where a large number of these immature and non-functioning synapses are continuously eliminated while a subset of active synapses are maintained and strengthened (Hua and Smith 2004; Huberman et al. 2008; Katz and Shatz 1996).

Chemical neurotransmission is the major mode of neuronal communication. This process consists in the release of neurotransmitters from neurons that act on receptor sites typically present on the membranes of postsynaptic cells and are able to trigger functional changes. Several molecules serve as neurotransmitters, such as acetylcholine, glutamate, aspartate, γ-aminobutyric acid (GABA) and glycine, and their release at the pre-synaptic terminal is induced by calcium (Ca^{2+})-regulated vesicle exocytosis. Glutamate is the major excitatory neurotransmitter in the CNS and glutamate receptors are widespread in the nervous system, mediating the vast majority of excitatory synaptic transmission in the brain. The main types of post-synaptic receptors for glutamate are: a) ionotropic glutamate receptors that are ligand-gated ion channels and are divided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptors; and b) metabotropic glutamate receptors that are receptors coupled to guanosine triphosphate (GTP)-binding proteins. However, excessive activation of these receptors under pathological conditions, due to increased concentrations of glutamate in the synaptic cleft, can induce the accumulation of intracellular Ca^{2+}, which may culminate in neurodegeneration (Salinska et al. 2005).

1.2. Astrocytes

Astrocytes are the most numerous glial cell in the brain, comprising about 85% of the glial population. The designation refers to their stellate morphology observed in histological preparations, although it widely varies. These cells were classified into, at least, two main classes, protoplasmic and fibrillary astrocytes, based on the morphology, anti-genetic phenotype, and location. Protoplasmic astrocytes that have short, thick and highly branched processes, are found in grey matter and their processes envelope synapses, as well as blood vessels (Barres 2008; Bushong et al. 2002). Fibrillary astrocytes that have long, thin and less branched processes, are located in the white matter, and are able to contact nodes of Ranvier and blood vessels (Barres 2008). Therefore, astrocytes act as bridges among all kinds of cells in CNS, including neurons, OL, microglia, endothelial cells, and their own. In this context, some

astrocytic processes that surround blood vessels with their end-feet may control the function and integrity of the blood-brain barrier (BBB), known to protect the CNS from unwanted substances in the general circulation (Gordon et al. 2007; Iadecola and Nedergaard 2007; Metea and Newman 2006). Other astrocytes contact with individual synapses and modulate synaptic transmission through their ability to regulate the local concentrations of ions and neurotransmitters (Huang and Bergles 2004; Schousboe 2003; Seifert et al. 2006; Wallraff et al. 2006). In addition, because astrocytic processes are highly dynamic subcellular elements capable of mobility, retraction and extension, astrocytes can shape the extracellular space having a strong impact on the neuronal network by influencing the extracellular diffusion of neurotransmitters (Sykova and Nicholson 2008; Theodosis et al. 2008). Since a single astrocyte may be in contact with thousands of synapses, and astrocytes communicate between them, another important feature of astrocytes on neuronal networks is their ability to simultaneously activate distinct populations of neurons (Angulo et al. 2004; Fellin et al. 2009).

In the last years, several studies have revealed that astrocytes play an important role in events of brain development, such as proliferation and differentiation of neuronal precursors (Kornyei et al. 2005; Lim and Alvarez-Buylla 1999; Song et al. 2002; Spohr et al. 2008), neuronal migration (Hatten 1999), axonal guidance (Martinez and Gomes 2002; Martinez and Gomes 2005) and myelination (Ishibashi et al. 2006).

Additionally, astrocytes participate in pathological conditions. In those cases astrocytes often withdraw their arms and slack off on their stabilizing chores, resulting that their role as neuronal partners become weaken or even disappear. However, they can release neurotrophic factors, like the transforming growth factor β (TGF-β) and the nerve growth factor (NGF), with beneficial roles in repairment. In addition, upon a CNS lesion, astrocyte proliferation is promoted in order to fill up the space left by the cells that cannot regenerate and form the glial scar (Escartin and Bonvento 2008). In this context, astrocytes suffer a morphological transformation, acquiring a fibrillary conformation, with longer slender processes and increased production of the cytoskeletal protein glial fibrillary acidic protein (GFAP) (Hadley and Goshgarian 1997).

1.3. Microglia

Microglia are specialized macrophages that can be found postnatally in all regions of the CNS, being distinguished from other glial cells by their origin, morphology, gene expression pattern and functions (Kettenmann et al. 2011; Ransohoff and Perry 2009). Microglia constitute approximately 10% of CNS glia and are considered the resident macrophages that serve both glial and immune-related

functions (Hanisch and Kettenmann 2007; Lawson et al. 1990; Soulet and Rivest 2008). In contrast to macroglia, which derive from ectodermal precursors within the embryo nervous system, microglia is originated from bone marrow precursors (Kaur et al. 2001) and migrate to the CNS during the embryonic development (McKercher et al. 1996; Monier et al. 2007), thus being considered as the first line of defense. After invading the brain parenchyma, microglial cells transform into the ramified phenotype after the first weeks of postnatal life. Like their peripheral macrophage counterparts, microglia display a remarkable range in both morphology and activity depending, in part, on the functionality of the surrounding tissue (Lynch 2009; Ransohoff and Perry 2009). Microglia can be classified into at least two morphologically distinguishable configurations: resting/surveilling and activated. Resting/surveilling microglia is present in the healthy matured tissues of the brain, presenting a ramified morphology with a small soma and fine cellular processes, which does not suggest an immediate association with a macrophage nature (Kettenmann et al. 2011). During several years it was thought that resting microglia were functionally dormant with an immobile ramified morphology, but more recently some studies demonstrated that this microglia are extremely dynamic, changing their morphology by extending and retracting highly motile processes in order to surveil the microenvironment in CNS, acquiring the designation of surveilling microglia (Davalos et al. 2005; Nimmerjahn et al. 2005). Interestingly, some studies have demonstrated that these microglia are implicated in developmental synaptic pruning in several brain regions during postnatal synaptic remodeling and in modulation of synaptic activity (Bessis et al. 2007; Coull et al. 2005; Dalmau et al. 1998; Fiske and Brunjes 2000). Recent research suggest that microglia-mediated engulfment of developing synapses is mediated by an interaction between the phagocytic complement receptor expressed on the surface of microglia and its ligand C3, a complement protein that is enriched at developing synapses (Schafer et al. 2012).

In other hand, when brain homeostasis is lost, microglial activation is accompanied by marked morphological changes. In fact, activated microglia revert to an amoeboid appearance by reducing the complexity of their shape with consequent retraction of their processes, as observed following staining for ionized calcium binding adaptor molecule (Iba)-1 (Imai et al. 1996; Imai and Kohsaka 2002), and increased expression of cellular markers such as cluster of differentiation (CD) 68 or CD11b (Liaury et al. 2012; Verney et al. 2010). Microglia also undergo rapid proliferation to increase the protection of other cells in the CNS (Kim and de Vellis 2005). Microglial activation may start with an early emergency response, such as defense oriented functions, to fight off an infection, or to limit further damage after an injury. In this

condition microglia can unfold their phagocytic activity in order to engulf cellular tissue debris, damaged cells or microbes. However, excessive microglial activation may result in increased production of many pro-inflammatory mediators, like cytokines, chemokines, reactive oxygen species (ROS) and NO, that may result in pathological forms of inflammation thus contributing to the progression of neurodegenerative and neoplastic diseases (Glass et al. 2010; Perry et al. 2010).

1.4. Oligodendrocytes

OL are the myelinating cells of the CNS that constitute about 5 to 10% of the total glia population. These cells extend many processes and each one contact and envelopes a stretch of axon, forming one of the most specialized cellular structures in the body, the myelin sheath (Bunge et al. 1962; Bunge 1968). The myelin sheath forms electrical insulation around nerve fibers making possible the rapid and saltatory transmission of the electrical impulse along the axon. In unmyelinated electrically active fibers a high consumption in adenosine triphosphate (ATP) takes place in order to restore the ion gradients. In contrast, in myelinated axons there is a strong reduction in energy consumption, since the action potentials and ion currents are restricted to less than 0.6% of the axon's surface (Nave 2010). Each OL is able, depending on its location, to myelinate up to 40 axons but on the same axon, adjacent myelin segments belong to different OL (Baumann and Pham-Dinh 2001). In addition, OL synthesize a number of trophic factors that contribute to neuronal survival and to the maintenance of axonal integrity, while neuron-OL interactions have been shown to influence neuronal size and axon diameter (McTigue and Tripathi 2008; Nave 2010). In this context, once peroxisomes are necessary for fatty-acid-β-oxidation, OL are crucial for axon function (Nave 2010). Moreover, the protein 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), present in non-compact myelin (Gravel et al. 2009), although not essential for myelination, is required for axonal integrity (Lappe-Siefke et al. 2003). Some other studies have shown that a subset of progenitor cells from the OL lineage, expressing the proteoglycan NG2, are distributed throughout the brain and are able to form excitatory and inhibitory connections with glutamatergic neurons, contributing actively and directly to neuronal signalling. The maturation of these cells leads to a rapid removal of the synaptic input, suggesting that only progenitor cells are able to monitor the firing patterns of surrounding neurons (De Biase et al. 2010).

2. Oligodendrocytes during central nervous system development

2.1. Oligodendrocyte origin

OL development is better understood in the spinal cord (SC) than in the brain. The human OL lineage has been characterized mainly in first trimester SC, while for human cerebrum the studies mostly refer the second and third trimester.

Since OL are evenly distributed throughout the adult CNS, it would be reasonable to suppose that they are produced from all regions of the neuroepithelium. However, several studies demonstrated that, in both SC and telencephalon, OL originate from specific regions. Embryonic oligodendroglial specification shares mechanistic features with motor neurons of the ventral neural tube (Kessaris et al. 2001; Rowitch 2004). A critical step during this process is the establishment of distinct progenitor cell domains (Jessell 2000; Marquardt and Pfaff 2001). In the SC, most OL derive from a specialized domain of the ventricular zone, called motor neuron precursors (pMN) domain, which gives rise to motor neuron precursors and then to oligodendrocyte precursor cells (OPC) (Lu et al. 2002; Sun et al. 1998; Takebayashi et al. 2002; Zhou and Anderson 2002). pMN progenitors develop in stages, after the first motor neuron formation, that is completed at embryonic day (E) 10.5 in mice, a phase of OL production starts at E12.5. This process depends on Sonic hedgehog homolog (Shh) signalling that acts through activation of Olig2, a transcription factor that is essential for OL development (Lu et al. 2000). Interestingly, it seems that during early fetal stage OL develop only from ventrally derived progenitor cells, since it was demonstrated that when E14 rat SC are divided into dorsal and ventral regions and the cells cultured separately, OL develop only in ventral cultures (Hall et al. 1996; Warf et al. 1991). OPC derived from the pMN continue to proliferate after specification and migrate both laterally and dorsally to occupy all areas of the SC. At a later fetal phase (starting around E15.5), an additional source of OPC arises in the dorsal spinal cord, contributing to 10-15% of the final OL population in the SC (Cai et al. 2005; Fogarty et al. 2005; Vallstedt et al. 2005).

In the forebrain, the formation of OL is a process even more complex, with multiple waves of OPC production from embryonic to postnatal stages that emerge in a ventral-to-dorsal progression (Kessaris et al. 2006). At E12.5, the first wave of OPC develops from Nkx2.1-expressing precursors in the ventricular zone of the ventral medial ganglionic eminence and anterior entopeduncular areas. Subsequently they migrate to all parts of the telencephalon, entering the cerebral cortex at E16. A second wave of OPC from Gsh2 expressing precursors occurs around E14.5 from the lateral and/or caudal ganglionic eminences. Finally, a third wave within the postnatal cortex

from Emx1-expressing cortical precursors starts around birth at post-natal day (P) 0 (Kessaris et al. 2006). After this process, OPC exhibit multidirectional migration in the ventricular zone to distant sites under control of several repulsive and attractive cues (Simpson and Armstrong 1999; Tsai et al. 2002; Tsai et al. 2003). Upon reaching their destination, progenitors begin to differentiate increasing the number and complexity of their processes.

2.2. Temporal oligodendrocyte lineage progression

While the production and migration of neurons occurs mainly during the prenatal period, the proliferation and migration of glial cells are essentially postnatal processes that last for an extended period after birth, with differentiation and maturation taking place throughout childhood. Given these overlapping situations there are several doubts about the temporal extent of each glial cell process during the postnatal period in humans, as well as their intermingled phases.

The progression along oligodendroglial lineage occurs through a serie of differentiation steps that can be identifiable accordingly to their migratory capacity, the dramatic increase in morphologic complexity and the expression pattern of specific markers (Fig. I.2). During maturation, oligodendroglial cells acquire an elaborate morphology and lose their capacity to proliferate and migrate. Briefly, the majority of authors divide OL maturation stages in four different steps: OPC, preoligodendrocytes (or late OPC), immature (or pre-myelinating) OL and mature (or myelinating) OL. OPC are proliferative cells, with a great migratory capacity and a specific expression of platelet-derive growth factor receptor α (PDGF-Rα), of the ganglioside A2B5 and of the proteoglycan NG2 (Nishiyama et al. 1996; Pringle et al. 1992). Although NG2 progenitors evidence slightly different morphologies depending on their location in the brain, they are usually characterized by a small polygonal soma and a multipolar tree of fine processes (Bergles et al. 2000; Gallo et al. 2008; Kukley et al. 2010). However, some studies demonstrated that NG2 cells in white matter have a bipolar morphology characteristic of neural precursor cells, with only few processes that are short in length and emanate from the opposing poles of the cell body (Chittajallu et al. 2004). During the differentiation process, OPC give rise to preoligodendrocytes that extend multipolar short processes and start to express, in addition to OPC markers, the sulfatide recognized by the O4 antibody (Sommer and Schachner 1981), which expression persists until the immature OL stage. Upon loss of the A2B5 and NG2 markers, the immature OL continues to express O4 and starts the expression of galactocerebroside C (GalC) (Yu et al. 1994). At this point OL become post-mitotic cells with long ramified

branches, committed to the oligodendroglial lineage (Armstrong et al. 1992; Gard and Pfeiffer 1989). In the final stages of OL development, the cells reach the mature OL stage by extending membranes that form compact enwrapping sheaths around the axons and by expressing, in an orderly manner, myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), and finally myelin oligodendrocyte glycoprotein (MOG) (Reynolds and Wilkin 1988; Scolding et al. 1989; Zhang 2001).

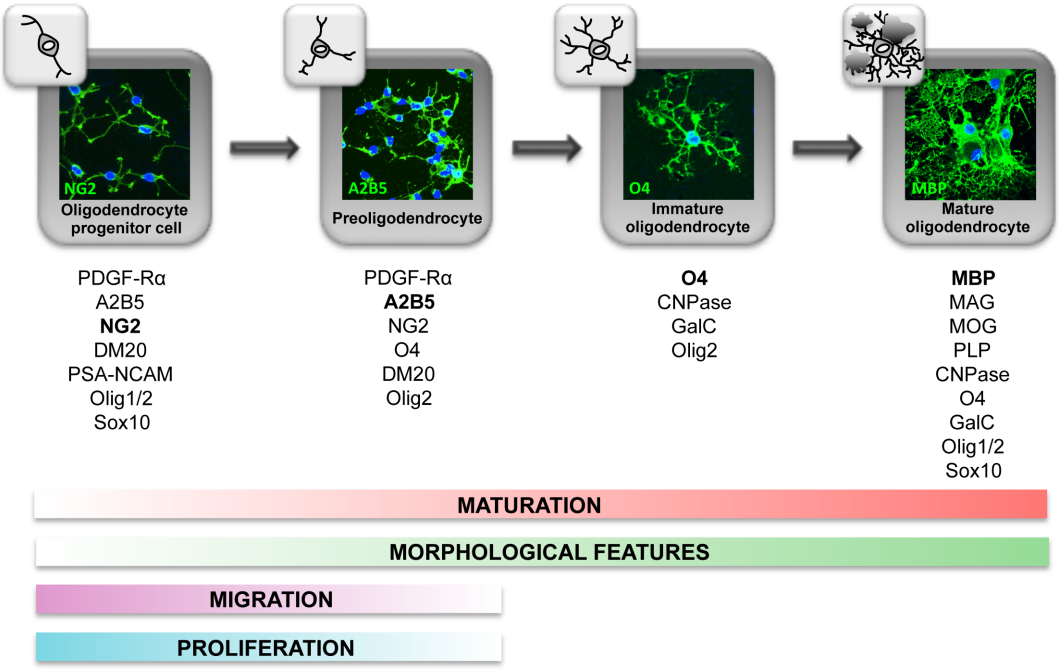


Fig. I.2 – Four steps of oligodendrocyte maturation towards oligodendroglial lineage: oligodendrocyte progenitor cells, preoligodendrocytes, immature oligodendrocytes and mature oligodendrocytes. These stages are identifiable accordingly to their increasingly complex morphology, the expression pattern of well-defined markers and their ability to proliferate, migrate and differentiate. CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GalC, galactocerebroside C; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PDGF-Rα, platelet-derived growth factor; PLP, proteolipid protein; PSA-NCAM, polysialic acid-neural cell adhesion molecule.

In humans, only a few studies have assessed the temporal OL development. These studies characterized four successive OL stages, the OPC, preoligodendrocytes, immature OL and mature OL characterized between 10 and 41 gestational weeks. The first OPC are observed in the forebrain at 10 weeks, but they appear in higher numbers only around 15 weeks, when they are most numerous in the ganglionic eminences and in the cortical ventricular zone/subventricular zone (Jakovcevski et al. 2009). Between 18 and 28 weeks, while OPC and preoligodendrocytes, identified by NG2 staining, are the major OL stages, immature OL represent a minor population. Between 28 and 40 weeks, a wave of OL differentiation

is accompanied by an increase in the number of immature OL and in MBP+ myelin sheaths that are restricted to the periventricular white matter (Back et al. 2001; Craig et al. 2003). Approximately at the 30 weeks of gestation a marked increase in O4+ cells displaying a complex multipolar morphology is observed mostly in deeper and milder cerebral white matter, while they are sparsely distributed in the superficial white matter, and not detected in cerebral cortex. Overall, the first MBP+ cells are observed between 20 and 28 weeks in subcortical regions, but are only extensively visualized between 36 and 40 weeks, with an increase from 1 to 5% in total brain volume that contains myelinated white matter (Back et al. 2001; Huppi et al. 1998).

In rodents, both rat and mouse present the first wave of OPC from E9.5 localized at the telencephalon, namely in the entopeduncular area (Timsit et al. 1995). In accordance, it was shown that in E13 mouse, ventral telencephalic regions have a greater capacity to generate OL *in vitro* than the corresponding cortical regions (Birling and Price 1998). At P2, both rat and mouse evidence preoligodendrocytes as the major OL stage in the cerebral white matter being immature OL a minor population. Lately at P7, the white matter contains now more than 80% immature OL, that begin to initiate myelination (Dean et al. 2011).

In comparison (Fig. I.3), OL lineage progression in the P2 rodents is similar to that of humans between 18 and 27 weeks in that cerebral white matter, being mostly composed by preoligodendrocytes and few immature OL. Later at P7, the white matter from rodents presents a state of maturation similar to the one observed in human between 30 and 36 weeks (Craig et al. 2003). Finally, the first MBP+ cells are observed around P7 rodents, becoming increasingly abundant at P14, both in the rat and mouse brain (Bjelke and Seiger 1989; Hardy and Friedrich 1996; Hartman et al. 1979), what is similar to the extent of myelination in many full-term infants (Back et al. 2001).

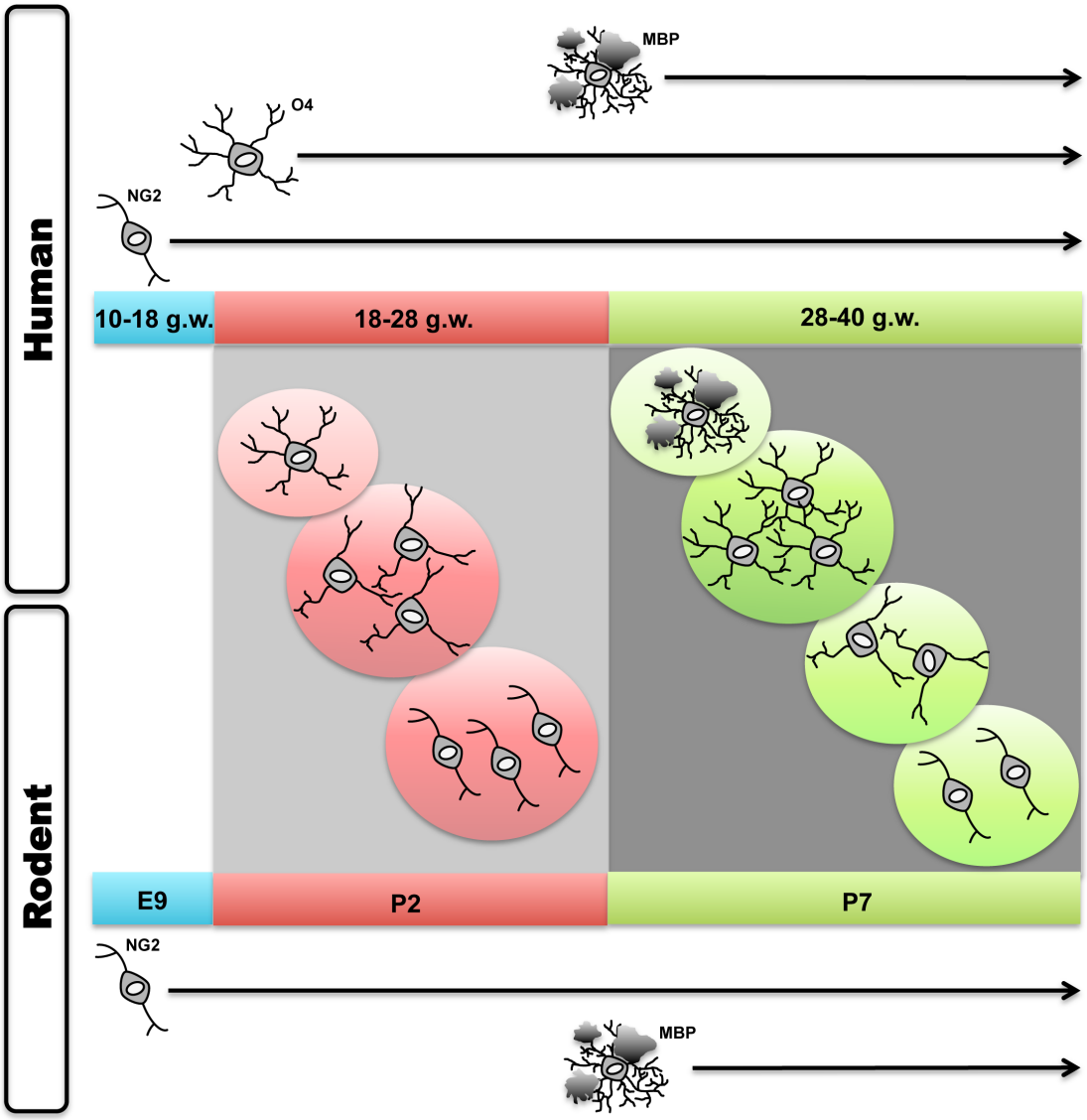


Fig. I.3 – Comparison between oligodendrocyte lineage development in human and rodents. Oligodendrocyte precursor cells (OPC), identified by NG2 staining, appear around embryonic day (E) 9 in rodents and between 10 and 18 gestational weeks (g.w.) in humans. Oligodendrocyte lineage at postnatal day (P) 2 in rodents is similar to that in humans between 18 and 28 g.w. and is composed mainly by OPC and preoligodendrocytes, with a minor population of immature oligodendrocytes (O4+ cells, see Fig. I.1). White matter at P7 rodents is similar to that observed in humans between 28 and 40 g.w., with predominance of immature oligodendrocytes and a progressive increase in mature oligodendrocytes that express the myelin basic protein (MBP).

2.3. Myelination

Myelination typically proceeds in a posterior to rostral direction (Coffey and McDermott 1997). Some degree of myelination is observed prenatally in humans, so that the peripheral nerves, the pons, and the cerebellar peduncles are fairly well myelinated at birth (Wiggins 1982). At 6 months, the genu of corpus callosum is myelinated and, thereafter, between 8-12 months myelination takes place in frontal,

parietal and occipital lobes (Paus et al. 2001). The volume of white matter increases progressively by approximately 12% from 4 to 22 years of age, but in the prefrontal cortex, myelination is only completed in adolescence/early adulthood (Woo and Crowell 2005). In comparison to human, some studies have revealed that rat myelination appears to begin in the caudal/brainstem regions and to proceed to the forebrain, involving medial regions before the lateral ones, as in humans (Coffey and McDermott 1997).

Myelination is a multi-step process that involves the recognition and adhesion of OL to the appropriate axon, the synthesis and transport of myelin components to the OL outer membrane, the wrapping of the myelin membrane around the axons and the compaction of the myelin sheath. OL have to synthesize a tremendous membrane amount in a short period of time. In fact, each OL is able to produce 40 myelinated axons (Pfeiffer et al. 1993). Myelin sheath is a fatty spiral insulation composed by extensions of modified plasma membrane of OL that surrounds the axons. Moreover, myelin is a multilayered stack of membranes that are tightly attached to the OL cytosol and their external surface. This structure is able not only to insulate the axon, but also to cluster Na^+ channels into the nodes of Ranvier enabling the action potential to jump from one node to the other (Waxman 2006). The myelin assembly and saltatory nerve conduction allow the fast processing of information in a relatively small space.

Myelin besides mostly containing cholesterol is also constituted by phospholipids and glycolipids (Baumann and Pham-Dinh 2001). In addition, OL and myelin lipids are rich in glycophingolipids, in particular galactocerebrosides, like GalC, and their sulfated derivatives (Raff et al. 1978; Zalc et al. 1981). Regarding protein content, the major CNS myelin proteins are MBP and PLP, which constitute approximately 80% of total proteins (Baumann and Pham-Dinh 2001), although two other minor glycoproteins denominated MOG and MAG are also considered important for myelin structure.

2.4. Regulation of oligodendrocyte development and myelination

Oligodendrocyte development is orchestrated by an extremely complex program that involves several factors with distinct roles at each step. These signals serve two major purposes: 1) help to control the proper timing of OPC differentiation to ensure myelination at the appropriate moment and place, and 2) control and match the number of OL to the axonal surface area requiring myelination.

2.4.1. Transcription factors

Part of the complexity attributed to myelination derives from research in recent years, which revealed a complex regulatory network of transcription factors in OL development. Until now, many transcription factors have been implicated, from lineage specification through their progressive stages of maturation until myelination (Gokhan et al. 2005; Stolt et al. 2006). However, the best knowns are two families of transcription factors: Sox group (Sox8, Sox9 and Sox10) and Olig genes (Olig1 and Olig2) (Liu et al. 2007). Regarding to Sox family, Sox9 is involved in OL specification, while Sox 8 is required for the terminal differentiation, and Sox10 is necessary for the development of myelin-forming OL (Stolt et al. 2004; Woodruff et al. 2001). The two Olig genes besides structurally similar and coordinately expressed, encode proteins with quite distinct biological capabilities. Expression of Olig2 plays prominent roles in multipotent neural progenitor cells of the embryo and adult being necessary for OL lineage development. Indeed, Olig2 is required for the development of NG2+ progenitor cells (Copray et al. 2006; Lu et al. 2002). Interestingly, Olig2 is strongly upregulated during acute brain damage (Buffo et al. 2005), what may indicate an increase in OL proliferation to counteract defects in the number of OL and myelination. On the other hand, Olig1 appears to be mostly implicated in OL maturation, being involved in the final stages of myelin production (Lu et al. 2002; Xin et al. 2005) and regeneration (Arnett et al. 2004).

2.4.2. Cytoskeleton components and their regulation

As described before, OL suffer continuous remodeling of the cytoskeleton in order to be able to extend their processes and ensheath the axons. Changes in OL shape are in part mediated by the cytoskeleton that is composed by microtubules (MT) and microfilaments (MF). These elements have distinct roles; while MT confer mechanical stability to OL processes, MF mediate process outgrowth and basic stability, as a consequence of their localization immediately beneath the plasma membrane.

MT are composed by heterodimers of α- and β-tubulin protein subunits that are anchored in the MT organizing center in the vicinity of the nucleus and extend to the OL periphery, giving origin to filaments arranged in parallel to the main axis of the process (Lunn et al. 1997; Simpson and Armstrong 1999). More recently, the importance of cytoskeleton during OL development was emphasized by the discovery of a specific form of β-tubulin, the β_{IV}-tubulin that has not yet been found in other CNS cells (Terada et al. 2005). Tubulin undergoes several posttranslational modifications,

including α-tubulin acetylation that is correlated with higher stability in more mature OL (Song et al. 2001). So, to increase MT dynamics during OL maturation and myelination, tubulin must be deacetylated in a process mediated by silent information regulator type 2 (Li et al. 2007).

Regarding to MF, actin is present in OL in two different states, as globular monomers (G-actin) or as filamentous polymers (F-actin). *De novo* actin nucleation to form G-actin is a kinetically unfavorable process due to the extreme instability of these small action oligomers, but it is thought that both actin-related protein 2/3 (Arp2/3) complex and formins could be involved as stabilizers (Pollard 2007). In this context, it is known that Arp2/3 complex is activated by cortactin and by Wiskott-Aldrich syndrome protein (WASP) family proteins (Weaver et al. 2003). These proteins polymerize actin monomers into F-actin filaments to generate small membrane protrusions for filopodia and lamellipodia formation (Bretschneider et al. 2004; Mullins et al. 1998; Svitkina and Borisy 1999). Filopodia are narrow structures supported by tightly packed parallel actin bundles with their plus ends facing the membrane, whereas lamellipodia are broader and contain actin networks arranged in an approximately orthogonal manner (Bacon et al. 2007; Sloane and Vartanian 2007).

In other hand, WASP proteins are themselves controlled by the Rho family GTPases that regulate cytoskeletal structure by mediating actin polymerization (Begum et al. 2004; Stradal et al. 2004). The Rho GTPases are divided in 8 subfamilies that include the RhoA-related subfamily (RhoA, RhoB, RhoC), the Rac1-related subfamily (Rac1, Rac2, Rac3, RhoG) and the Cdc42-related subfamily (Cdc42, TC10, TCL, Chp/Wrch-2, Wrch-1). Rho GTPases are binary molecular switches that cycle between an inactive guanosine diphosphate (GDP)-bound and an active GTP-bound state in response to extracellular stimuli (Fig. I.4). In the CNS, active Rac1 and Cdc42 act as positive regulators of morphological differentiation, inducing process extension and branching, while RhoA acts as a negative regulator inhibiting process elongation (Liang et al. 2004). Regarding RhoA, B and C, they activate the immediate downstream Rho-associated protein kinase (ROCK), which in turn phosphorylates a number of actin cytoskeleton regulators, like the enzyme myosin light chain phosphatase and the myosin light chain (Maekawa et al. 1999). This direct phosphorylation increases the contraction of the actomyosin network (Amano et al. 2000; Riento and Ridley 2003). Rac1 and Cdc42 activate WASP family of proteins, like neuronal WASP (N-WASP) and Wiskott-Aldrich syndrome protein family verprolin homologs (WAVE) 1/2 (Derivery and Gautreau 2010; Kempiak et al. 2005; Symons et al. 1996; Takenawa and Miki 2001). These proteins, as mentioned, bind the Arp2/3 complex and alter its conformation for actin binding. WAVE1 and N-WASP are also critical for myelination, since deletion of

WAVE1 in mice causes hypomyelination, while the N-WASP inhibitor wiskostatin causes retraction of filopodia and lamellipodia and impairs myelination (Kim et al. 2006).

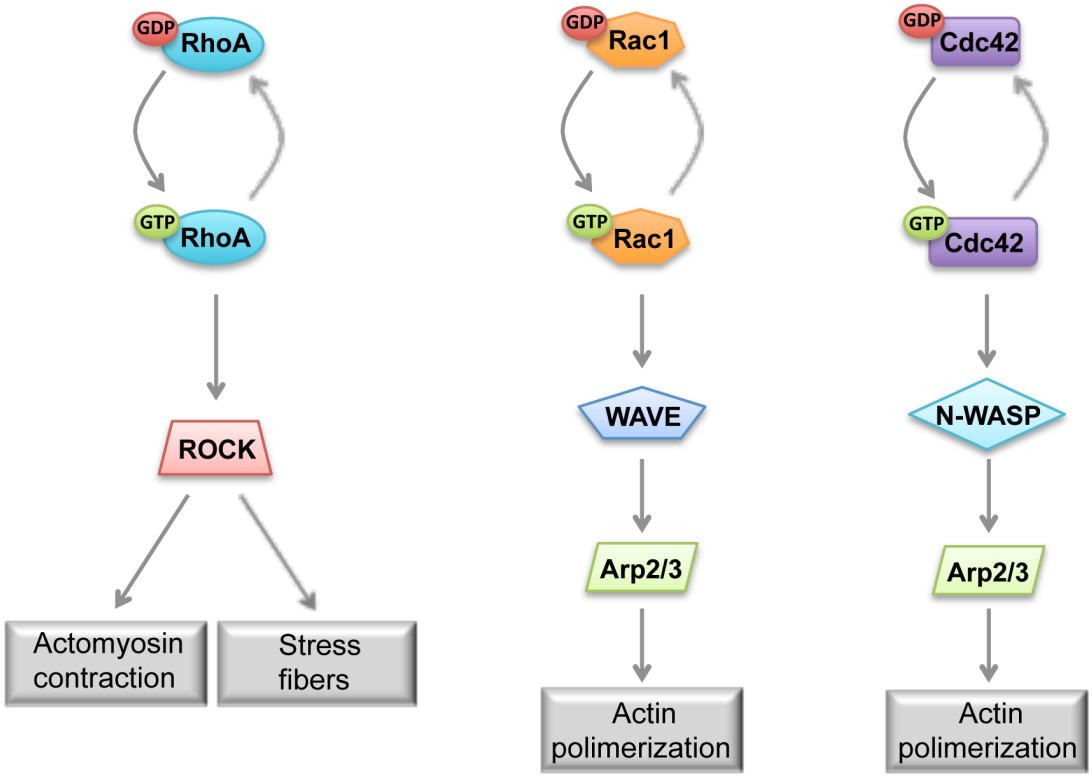


Fig. I.4 – Regulation of the actin cytoskeleton by Rho GTPases. Principal pathways regulated by RhoA, Rac1 and Cdc42. Active Rho A activates Rho-associated protein kinase (ROCK) leading to actomyosin contraction and stress fibers. On other hand, activated Rac1 and Cdc42 induce activation of Wiskott-Aldrich syndrome protein family verprolin homologs (WAVE) and neuronal Wiskott-Aldrich syndrome protein (N-WASP), respectively, leading to changes in actin-related protein 2/3 (Arp2/3) and consequent actin polymerization. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

2.4.3. Extracellular factors

Many studies point out the importance of extracellular factors released by neurons and glial cells to multiple stages of OL development.

2.4.3.1. Growth factors

Several lines of evidence have demonstrated the importance of growth factors in OL development; while some promote the maintenance of the OPC pool, others induce OPC differentiation into myelinating OL. Moreover, one mechanism that may determine the final number of OL is the competition for limiting amounts of factors, like platelet-derived growth factor A (PDGF-A), fibroblast growth factor 2 (FGF-2), insulin-

like growth factor 1 (IGF-1), neurotrofin 3 (NT-3) and ciliary neurotrophic factor (CNTF) (Baron et al. 2005; Barres and Raff 1994; Miller 2002). PDGF-A is a potent mitogen produced by both astrocytes and neurons that regulates the proliferation and survival of OPC, preventing premature differentiation (Noble et al. 1988; Raff et al. 1988; Richardson et al. 1988) and inducing early OPC to proliferate for an indefinite number of divisions *in vitro* (Tang et al. 2001). FGF-2 is also a mitogen that stimulates proliferation of early and late progenitors, maintaining the expression of the PDGF-Rα and blocking the differentiation into OL (Bansal and Pfeiffer 1994; McKinnon et al. 1990). In addition, FGF-2 induces withdrawal of myelin sheets and downregulation of the major myelin proteins, at both the protein and messenger ribonucleic acid (mRNA) levels (Cohen and Chandross 2000; Fressinaud et al. 1995; Goddard et al. 2001; Grinspan et al. 1993; Hoffman and Duncan 1995). Although PDGF-A or FGF-2 acts individually with different effects on OPC, when jointly they induce continuous proliferation and produce a "conditional immortalization" of OPC (Bogler et al. 1990). IGF-1 in combination with FGF-2 and PDGF-A synergistically promotes deoxyribonucleic acid (DNA) synthesis in OPC (Jiang et al. 2001) and *in vitro* proliferation (McMorris et al. 1990). NT-3 induces OPC proliferation *in vivo* (Kumar et al. 1998; McTigue et al. 1998), but present a weak mitogenic effect *in vitro* (Yan and Wood 2000).

On the other hand, thyroid hormone (T3) is one of the best-characterized differentiation factors. This growth factor blocks OPC proliferation *in vitro* and induces their differentiation into OL even in the presence of PDGF-A (Baas et al. 1997; Barres et al. 1994; Ibarrola et al. 1996). In what regards myelination, T3 is necessary for proper timing and normal levels of myelin production *in vivo* and *in vitro* (Almazan et al. 1985; Calza et al. 2002; Dussault and Ruel 1987). Moreover, while increased concentrations of T3 as those observed in hyperthyroidism accelerate myelination, hypothyroidism results in its decrease (Walters and Morell 1981). However, it seems that this hormone is not essential for OL differentiation, but may be involved in regulating the moment of differentiation, since OPC cultured in the absence of mitogens stop dividing and differentiate rapidly, even in the absence of T3 (Barres et al. 1994). In other hand, in the presence of mitogens, T3 signalling is necessary to promote the complete differentiation of OPC (Baas et al. 2002).

2.4.3.2. Cytokines and chemokines

Cytokines are pleiotropic factors and most of them are secreted proteins or glycoproteins, while chemokines are small molecular weight cytokines, specialized in causing cell movement. However, both use chemical signals to induce changes in

other cells. Cytokines and their receptors are expressed physiologically in CNS cells and are important in the development and function of the brain, presenting several roles in different processes. Some cytokines involved in OL development include interferon (IFN)-γ, interleukin (IL)-1β, TGF-β, IL-6 and leukemia inhibitory factor (LIF). All of these cytokines, except the first, are able to inhibit OPC proliferation and enhance their differentiation (Mayer et al. 1994; McKinnon et al. 1993; Valerio et al. 2002; Vela et al. 2002). To note that, IFN-γ has exactly the opposite effect in OL development, inhibiting OPC differentiation and the cell cycle exit (Baerwald and Popko 1998; Chew et al. 2005). In addition, there is little evidence that OL produce chemokines, but it is known that in cell cultures CXC chemokines, such as growth-related oncogene-α, IL-8 and stromal cell-derived factor-1α, stimulate MBP production (Kadi et al. 2006), while astrocytic CXC ligand 1 in the SC enhances the proliferative response of OPC to PDGF-A (Robinson et al. 1998).

2.4.4. Neuron-oligodendrocyte communication

Since most of the factors described above are also produced by astrocytes the role of neurons on myelination was not initially apparent. In fact, neurons can direct the myelination of their axons, and consequently the OL differentiation, through neuronal-OL cross talk. It was initially thought that axon diameter represented the only and crucial regulator of myelination (Voyvodic 1989), with OL selecting axons with diameters above 0.2 μm and excluding dendrites (Simons and Trajkovic 2006). However, the molecular cues for this recognition remain unknown, it seems that besides axon diameter, also neurotransmission and cell adhesion molecules play an important role in OL differentiation and myelination (Fig. I.5).

2.4.4.1. Neurotransmitters and their receptors

As mentioned, the production of myelinated axons requires a precise matching of the number of OL generated to the length of axons to be myelinated. So, in order to detect the impulse activity through the activity-dependent release of molecules from axons, such glutamate and ATP, OL at different stages of development have to express ion channels as well as purinergic and other membrane receptors.

Both OPC and OL, express AMPA, kainate and NMDA receptors for glutamate. While AMPA/kainate receptors are predominantly expressed in the cell body, particularly in immature OL, NMDA receptors are mainly present in the myelinating processes (Bakiri et al. 2009; Butt 2006; Káradóttir and Attwell 2007). Interestingly, it

was reported that AMPA receptors in OL lack GluR2 subunit and therefore are Ca^{2+}-permeable, which has a crucial relevance for the damaging actions of glutamate on OL (Li and Stys 2000). However, it is not clear how glutamate signalling induces changes in OL maturation. Studies performed in *in vitro* cultures showed that glutamate response is more pronounced in OPC or immature OL than in mature OL (Deng et al. 2003; Itoh et al. 2002; Rosenberg et al. 2003). Furthermore, it seems that glutamate-evoked current do not generate different responses in OPC and mature OL (Berger et al. 1992; Ziak et al. 1998). In addition, studies with cells in culture and with organotypic slices demonstrated that activation of AMPA/kainate inhibits PDGF-induced proliferation and promotes OPC differentiation, leading to an increase of the cell size with the development (Káradóttir et al. 2005; Patneau et al. 1994). With regard to NMDA receptors, their preferential location in the distal process of OL suggests that they may have a role in controlling axon-OL interactions (Káradóttir et al. 2005; Micu et al. 2006; Salter and Fern 2005).

OL in different stages of maturation also express ATP-gated P_{2x7} receptors that are permeable to Ca^{2+}. Whereas glutamate has been mostly shown to regulate the early development of OL, adenosine and ATP are recognized as modulators of late OL development and myelination. It is believed that ATP, released by action potential firing, does not act directly on OL. Instead, the adenosine resulting from conversion of ATP by extracellular ATPases (Káradóttir and Attwell 2007) promotes an increase in OPC intracellular Ca^{2+}, inhibiting their proliferation and stimulating their differentiation, consequently promoting the myelin assembly (Othman et al. 2003; Stevens et al. 2002). In later development adenosine also acts indirectly by inducing astrocytes to release LIF, which enhances myelination by mature OL (Ishibashi et al. 2006).

As regards $GABA_A$ receptors, they are expressed in OL at different maturation stages (Matute et al. 1997; Pastor et al. 1995; Williamson et al. 1998) and GABA depolarizes both mature and progenitor cells. Moreover, OL precursors present in both grey and white matter receive GABAergic synaptic input from axons (Lin and Bergles 2004). In this context, GABA can activate $GABA_A$ receptors in OPC (Lin and Bergles 2004), inhibiting outward rectifying potassium channels (Pastor et al. 1995) that could lead to a reduction of proliferation.

2.4.4.2 Cell adhesion molecules

Another possible candidate for axonal signalling to OL that regulates myelination are the cell adhesion molecules. These molecules have the ability to bring the axon and glial cell into close apposition and to transduce the signals between those

cells (Doherty and Walsh 1996). The best-studied adhesion molecules are the polysialic acid-neural cell adhesion molecule (PSA-NCAM), L1 cell adhesion molecule and leucine-rich repeat- and Ig domain-containing nogo receptor-interacting protein 1 (LINGO-1). During development, the hemophilic NCAM-NCAM adhesion, i.e. cell-cell interaction (Bruses and Rutishauser 1998; Kiss and Rougon 1997; Rutishauser and Landmesser 1996), is prevented because all growing nerve fibers in the CNS express the PSA-NCAM (Charles et al. 2000), persisting in areas of adult brain that exhibit plasticity (Seki and Arai 1991; Seki and Arai 1993; Theodosis et al. 1998). So, in order to occur interactions between OL and neurons and consequently myelination, PSA-NCAM has to be downregulated when neurons are electrically active (Coman et al. 2005; Kiss et al. 1994; Landmesser et al. 1990). In a more recent study it was demonstrated that PSA-mediated signalling mechanism is one of the regulators of primary myelination in the human fetal brain (Jakovcevski et al. 2007).

L1, an adhesion molecule also expressed in axonal surface (Hortsch 1996; Hortsch 2000), is diffusely expressed on the non-myelinated axons, while it drastically reduces upon myelination, presenting low levels on myelinated axons (Jakovcevski et al. 2009). However, in this case, the L1 expressed in axons promotes myelination, probably acting at the very early stage of OL/axon adhesion, through binding possibly to a specific oligodendroglial receptor not yet identified. After an initial stage of adhesion, the downregulation of L1 from the axonal surface is necessary to the myelination onset and the beginning of wrapping process (Barbin et al. 2004).

LINGO-1, a transmembrane protein with leucine-rich repeats and an immunoglobulin domain, expressed in both OL and neurons, interacts with Nogo-receptor, and negatively regulates OL differentiation and myelination (Bourikas et al. 2010). Loss of LINGO-1 function in OL leads to increased myelination and its overexpression inhibits myelin assembly (Mi et al. 2005). LINGO-1 is also able to inhibit MBP transcription by constitutive inhibition of Fyn kinase, since the activation of this kinase is crucial for upregulation of MBP transcription during OL maturation (Mi et al. 2005; White et al. 2008). Although the molecular mechanism by which this protein influences membrane generation is not clear, it is known that LINGO-1 in OL inhibits process extension once it is a constitutive activator of Rho A (Erschbamer et al. 2005; Mi et al. 2005). In addition, the inhibition of LINGO-1 leads to the downregulation of RhoA activity thus promoting *in vitro* OPC differentiation (Mi et al. 2009; Zhao et al. 2007).

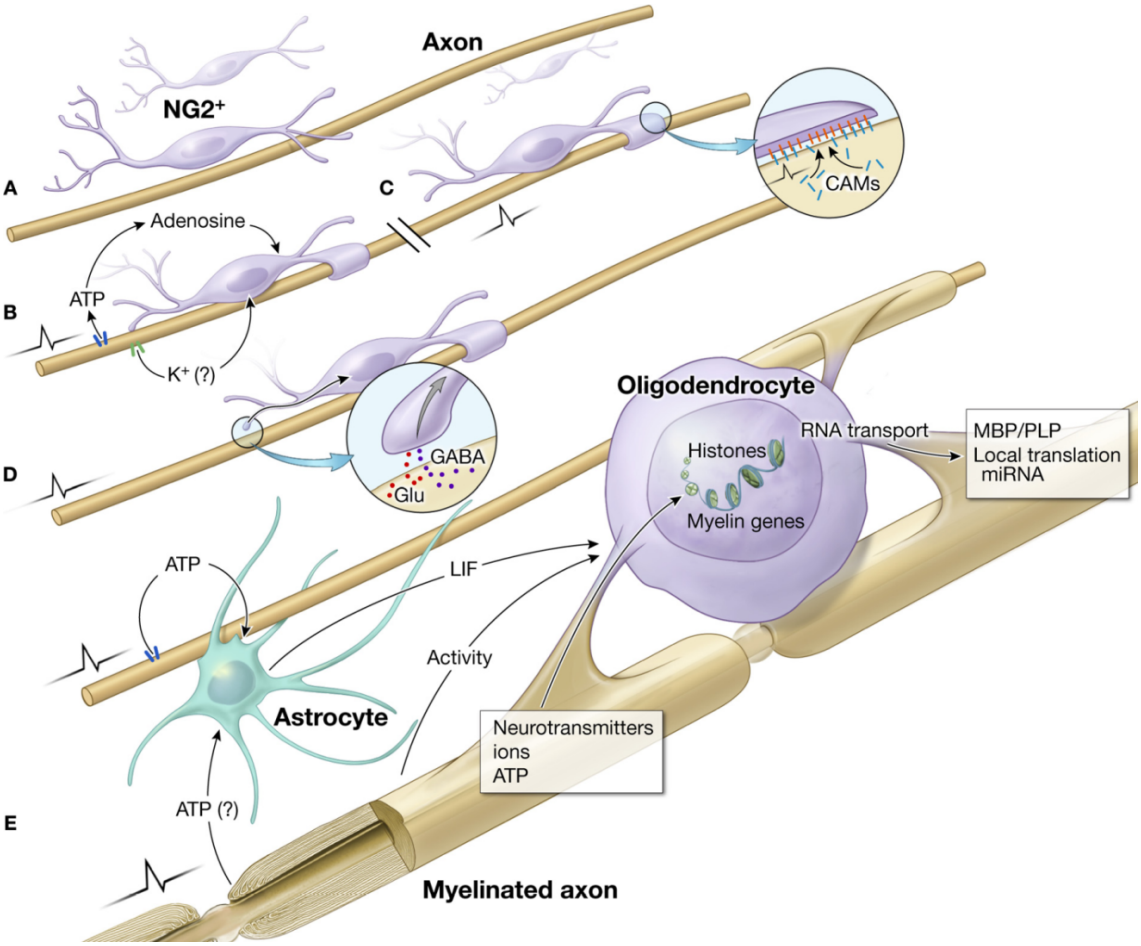


Fig. I.5 – Oligodendrocyte development and myelination are regulated by neuronal signals. a In absence of electrical activity oligodendrocyte precursor cells (NG2+ cells) do not differentiate and are not able to myelinate. **b** Electrical activity leads to adenosine triphosphate (ATP) release from axons, which in turn generates adenosine that induces oligodendrocyte maturation and myelination. **c** Electrical activity alters the expression of cell adhesion molecules on the axons, like polysialic acid-neural cell adhesion molecule (PSA-NCAM) and L1, that are involved in cell-cell interactions and consequently in myelination onset. **d** The release of neurotransmitters by neurons including glutamate and γ-aminobutiric acid (GABA) can also regulate oligodendrocyte maturation and myelination. **e** After oligodendrocyte precursor cell differentiation into mature oligodendrocytes, ATP release from axons will stimulate the release of leukemia inhibitory factor (LIF) from astrocytes, which in turn promotes myelination. From Lee and Fields 2009.

3. Mechanisms of oligodendrocyte injury

OL have a great metabolic rate in order to myelinate properly. Some studies pointed out that during myelination peak, OL produce three times its weight in myelin per day, and support membrane up to 100 times the weight of its cell body (Connor and Menzies 1996; Ludwin 1997; McLaurin and Yong 1995; McTigue and Tripathi 2008). This feature turns OL into cells highly vulnerable to several pathways of damage resulting from activation of numerous intracellular mechanisms, most of them produced by extracellular factors released by other CNS cells.

3.1. Molecular mechanisms

Among the molecular mechanisms that mediate OL damage, several authors indicate excitotoxicity, oxidative damage, endoplasmic reticulum (ER) stress and cytokine signalling as the key events.

3.1.1. Excitotoxicity

As described before, OL express several receptors such as AMPA, kainate and NMDA receptors that predispose them to excitotoxic cell death. In addition, OL also express the ATP receptor P_{2x7} that make them vulnerable to increased levels of extracellular ATP. OL are the predominant cells for glutamate clearance in human white matter, and in this context they express the excitatory amino acid transporter (EAAT)-1 and EAAT-2 (Bakiri et al. 2009; Matute et al. 2007a). In situations of ATP depletion these transporters revert their action, promoting changes in ion gradients, which leads to glutamate release from OL (Fern and Moller 2000; Li and Stys 2000).

The toxicity induced by glutamate and ATP primarily depends on excessive Ca^{2+} influx (Fig. I.6). All cells from OL lineage express NMDA receptors and their activation, upon increased extracellular glutamate, induce membrane depolarization and rise in cytosolic Ca^{2+} (Bakiri et al. 2009). Enhanced intracellular concentrations of Ca^{2+} lead to accumulation within mitochondria, and consequent depolarization of this organelle with increased production of ROS and release of proapoptotic factors that activate caspases (Matute 2006; Verkhratsky et al. 1998). The mechanisms triggered by NMDA receptor activation in OL have not been studied in detail until now, but it is known that NMDA is preferentially located on the distal processes of OL. This fact renders the myelin sheaths one vulnerable target for excitotoxic insults, leading to osmotic swelling, loosening, and vacuolation (Matute et al. 2007a). Interestingly, several pathways are initiated by AMPA and kainate receptors, indicating that different intracellular domains are involved in death program execution (Matute et al. 2007a). When AMPA receptors are activated, apoptosis is induced by caspase-8 recruitment and activation of the extrinsic apoptotic cascade, which in turn leads to truncation of Bid protein, and amplification of the signal through activation of the intrinsic pathway, culminating in caspase-3 and poly(adenosine diphosphate-ribose) polymerase (PARP) activation. In addition, necrosis may be seen upon AMPA receptor activation (Matute 2006; Sánchez-Gómez et al. 2003). On the other hand, kainate receptor activation primes caspase-9 and -3 activation leading to the execution of the intrinsic apoptotic cascade (Matute 2006; Sánchez-Gómez et al. 2003). Regarding to ATP, when in excess, the sustained activation of P_{2x7} induces an increase in intracellular Ca^{2+} that

results in caspase-3 activation, chromatin condensation and cell death by apoptosis or necrosis depending on the intensity of the insult (Matute et al. 2007b).

Excessive cytosolic Ca^{2+} can activate other pathways, such as calpains, phospholipases, endonucleases and NO synthase with consequent NO production. The reaction of NO with superoxide leads to the production of peroxynitrite that may promote oxidative toxicity to OL (Li et al. 2005). Calpains are intracellular Ca^{2+}-activated cysteine proteases that can mediate necrosis or caspase-independent apoptosis. These proteases cleave cytoskeletal proteins and proapoptotic members of the Bcl-2 family, like Bax (Cao et al. 2003; Chen et al. 2001; Villa et al. 1998), thus facilitating the release of the apoptosis-inducing factor from mitochondria, presumably through proteolytic cleavage of a membrane anchor that retains this factor on the inner mitochondrial membrane (Polster et al. 2005). Other studies suggest that calpain activation can lead to cell death by necrosis due to lysossomal rupture and cathepsin-mediated cell death (Artal-Sanz et al. 2006; Artal-Sanz and Tavernarakis 2005; Yamashima 2004). Moreover, calpains can cleave numerous substrates including key components of the Ca^{2+} signalling system, like the plasma membrane Ca^{2+}-ATPase, leading to a decrease in Ca^{2+} removal from the OL cytoplasm (Paszty et al. 2002).

3.1.2. Oxidative damage

Several features are responsible for the high vulnerability of OL to oxidative damage. As described before, mitochondria depolarization by increased intracellular concentrations of Ca^{2+} leads to an increase of ROS production with deleterious effects in OL viability (Matute 2006; Verkhratsky et al. 1998). OL consume large amounts of oxygen and ATP in order to produce myelin, which leads to the formation of hydrogen peroxide and other reactive species with toxic properties to these cells (McTigue and Tripathi 2008). Once OL have low concentrations of the antioxidant glutathione, the endogenous antioxidant defense, they are more vulnerable to an increase in intracellular concentration of hydrogen peroxide (McTigue and Tripathi 2008; Thorburne and Juurlink 1996). On the other hand, OL have the largest intercellular stores of iron in the brain (Cheepsunthorn et al. 1998; Thorburne and Juurlink 1996), since it is used as co-factor for myelin synthetic enzymes (Connor and Menzies 1996). Besides the critical role of iron in the myelin production (Todorich et al. 2009), when in the presence of hydrogen peroxide it may trigger the formation of hydroxyl radicals by the Fenton's reaction (Benarroch 2009). These radicals are very potent inducers of lipid peroxidation and, along with peroxidation products (e.g. 4-hydroxy-2-nonetal), are capable of impairing protein and acid nucleic functions, as well as membrane

destruction (Brito et al. 2008). Additionally, oxygen and NO radicals are particularly toxic to mitochondria through interaction and blockade of various proteins of the respiratory chain (Mahad et al. 2008; Smith and Lassmann 2002).

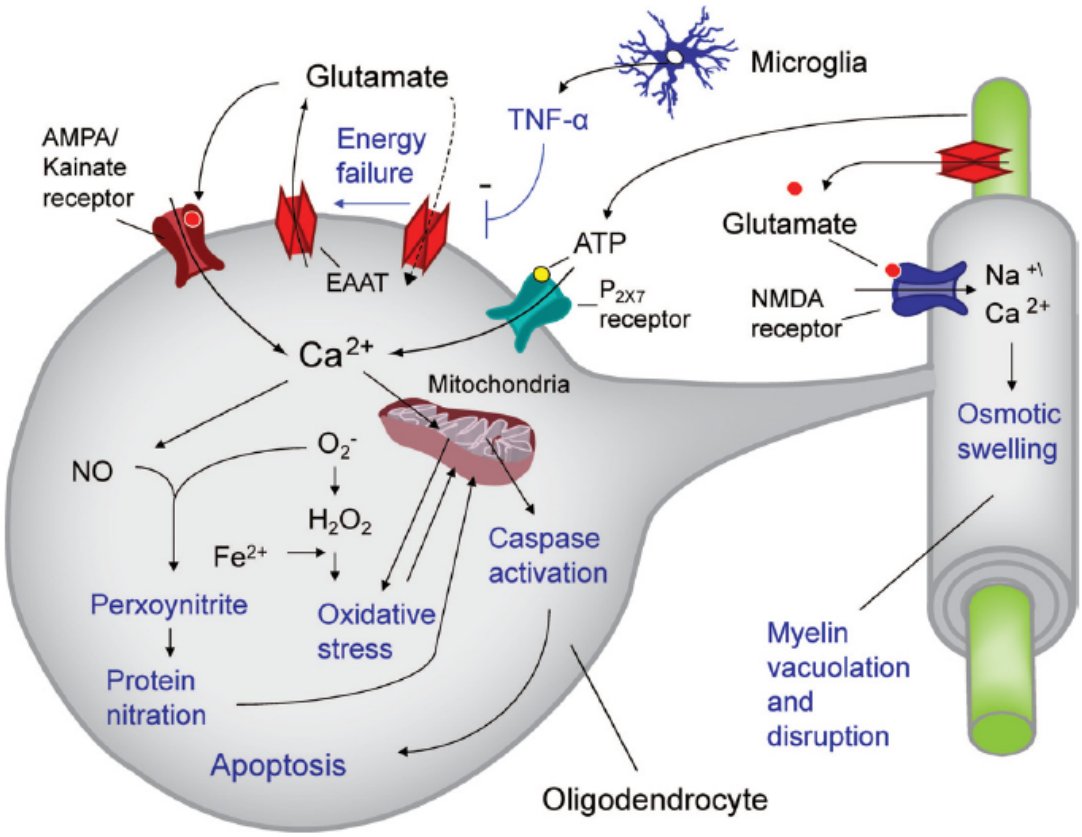


Fig. I.6 – Mechanisms of toxicity to oligodendrocytes. Primarily, glutamate- and adenosine triphosphate (ATP)-induced toxicity depends on excessive calcium (Ca^{2+}) influx, following activation of Ca^{2+}-permeable α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate, *N*-methyl-D-aspartate (NMDA) and ATP activated P_{2x7} receptors. Increased concentrations of intracellular Ca^{2+} lead to mitochondrial dysfunction and consequent reactive oxygen species production and caspase activation. On the other hand, iron (Fe^{2+}) can react with hydrogen peroxide (H_2O_2) with consequent production of hydroxyl radicals and oxidative stress. The preferential location of NMDA receptors on the distal processes of oligodendrocytes turns the myelin sheaths particularly vulnerable to insults that lead to osmotic swelling, myelin vacuolation and disruption. In addition, superoxide anion (O_2^-) reacts with nitric oxide (NO) leading to peroxynitrite formation that is toxic to oligodendrocytes. In addition, microglia release cytokines, such as the tumor necrosis factor α (TNF-α), that trigger and potentiate toxicity to oligodendrocytes. From Benarroch et al. 2009.

3.1.3. Endoplasmic reticulum stress

The ER is mainly recognized as a protein-folding factory, responsible for biosynthesis, folding, assembly and modification of several proteins (Kaufman 1999). However, during ER stress caused by accumulation of unfolded proteins or Ca^{2+} depletion, ER initiates the unfolded protein response (UPR) (Verkhratsky 2005), in order to ensure the fidelity of protein folding and prevent accumulation of these non-functional proteins (Fig. I.7). In this condition, cells alter their transcriptional and

translational programs to handle with stressful conditions and solve the protein-folding defects (Ron and Walter 2007; Schroder and Kaufman 2005). In mammalian cells, three ER-localized protein sensors initiate UPR signalling: inositol-requiring enzyme 1α (IRE-1α), pancreatic ER kinase (PERK) and activating transcription factor (ATF) 6 (Ron and Walter 2007; Schroder and Kaufman 2005). In the absence of ER stress, glucose-regulated protein (GRP) 78, also known as immunoglobulin-heavy-chain-binding protein (BIP), the best-characterized ER chaperone protein, is associated with the intraluminal regions of all these sensors assuring their inactive state. During ER stress, GRP78 is sequestered through binding to unfolded or misfolded proteins, leading to the release and consequent activation of the ER stress sensors (Bertolotti et al. 2000). So GRP78 has been used as a classical marker of UPR activation (Lee 2005). GRP78 dissociation leads to mobilization of ATF-6 for the Golgi and activation of IRE-1α and PERK through autophosphorilation. Activated IRE-1α induces X-box binding protein 1 (XBP1) splicing with the excision of a 26-nucleotide sequence and a consequent shift in the reading frame leading to the production of spliced XBP1 (XBP1s) (Calfon et al. 2002; Lee et al. 2002; Yoshida et al. 2001). After translation, XBP1s translocates to the nucleus and binds to UPR elements in order to induce several UPR genes that assist in protein synthesis and secretion, like ER-associated degradation (ERAD) protein, CCAAT/enhancer-binding protein homologous protein (CHOP) and $p58^{IPK}$ (Lee et al. 2003; Shaffer et al. 2004). Likewise XBP1s also induces lipid biosynthesis and ER biogenesis (Lee et al. 2008; Shaffer et al. 2004; Sriburi et al. 2004). In situations of ER stress, ATF-6 is translocated to the Golgi complex where it suffers cleavage (Chen et al. 2002; Ye et al. 2000). Its cytoplasmic ATF-6α fragment is released and then translocates to the nucleus in order to activate the transcription of target genes, such as GRP 78, CHOP and XBP1, leading to a redundant pathway (Hong et al. 2004). Finally, activated PERK initiates the phosphorylation, and consequent inactivation, of eukaryotic translation-initiation factor 2α (eIF2α), which in turns increases the expression levels of the transcription factor ATF-4 (Blais et al. 2004; Lu et al. 2004; Vattem and Wek 2004). ATF-4 translocation to the nucleus upregulates the transcription of UPR target genes, like CHOP, GRP78 and growth-arrest DNA damage gene 34 (GADD34) (Zinszner et al. 1998).

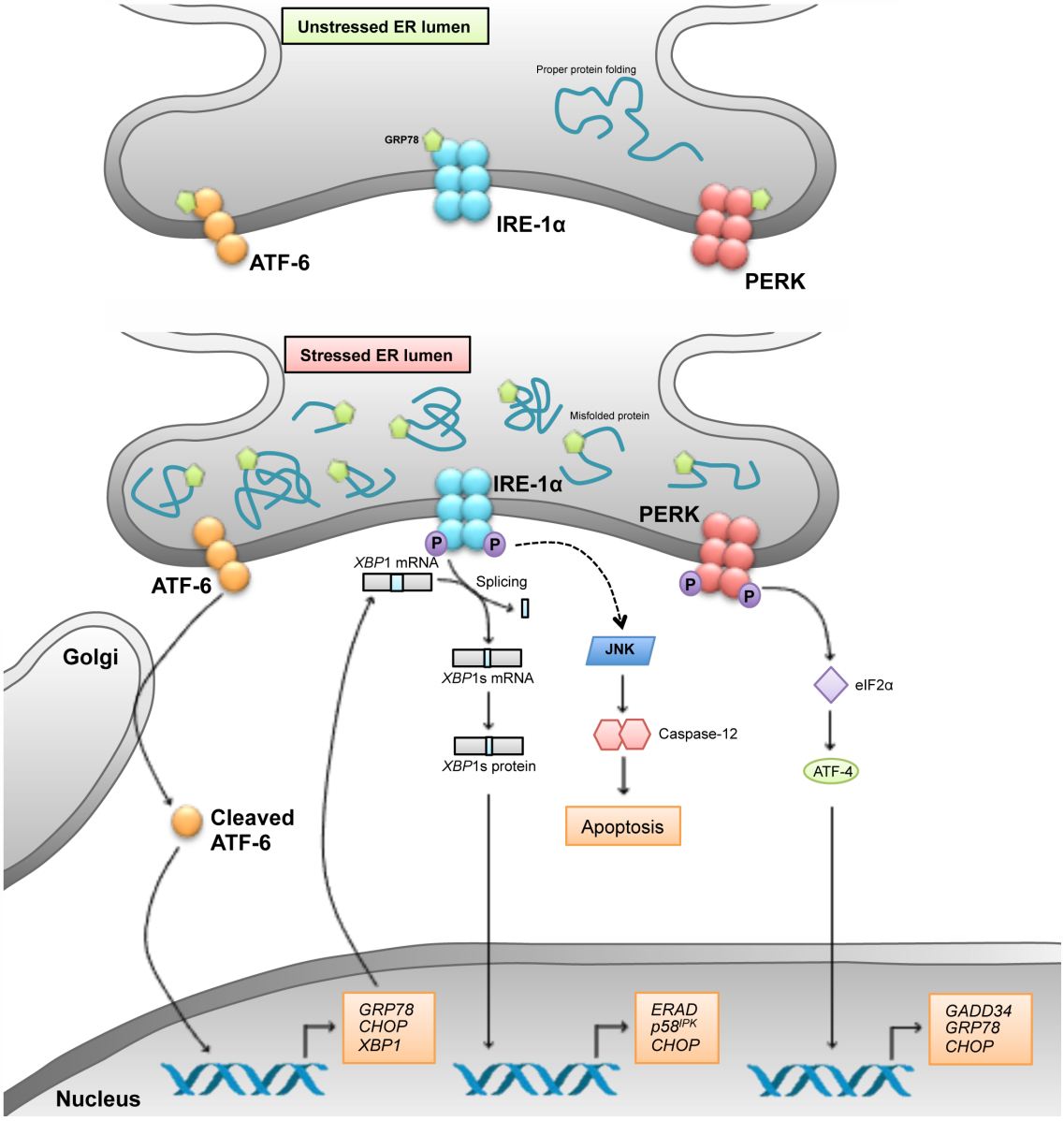


Fig. I.7 – Signal transduction events associated with endoplasmic reticulum (ER) stress. In conditions of unstressed ER (top panel), glucose-regulated protein (GRP) 78 binds to activating transcription factor 6 (ATF-6), inositol-requiring enzyme 1α (IRE-1α) and pancreatic ER kinase (PERK), thus preventing their activation. When unfolded proteins accumulate in ER lumen during ER stress (bottom panel), GRP78 is recruited away from ATF-6, IRE-1α and PERK, activating the different unfolded protein response (UPR) pathways. Release of GRP78 from ATF-6 allows the translocation of the protein to the Golgi apparatus for proteolytic processing and consequent release of active (cleaved) ATF-6, which controls expression of UPR genes. Activated IRE-1α results in spliced X-box binding protein 1 (XBP1s) production, which in turns induces the expression of genes involved in restoring protein folding or degrading unfolded proteins. Phosphorylated IRE-1α leads to activation of c-Jun *N*-terminal kinase (JNK), through TNF-α-receptor-associated factor 2 (TRAF2) binding, causing programmed cell death by caspase-12-mediated apoptotic pathway. Activated PERK results in phosphorylation of eukaryotic translation-initiation factor 2α (eIF2α) and consequent activation of ATF-4 that induces the expression of genes involved in restoring ER homeostasis. Adapted from Todd et al. 2008.

Accumulation of misfolded proteins in the ER can trigger Ca^{2+} release from this organelle, possibly through inositol-triphosphate receptors (Deniaud et al. 2008). The Ca^{2+} released from the ER is accumulated in mitochondria and causes its depolarization, disrupting electron transport chain and increasing ROS production (Gorlach et al. 2006). On the other hand ROS can in turn increase Ca^{2+} release from the ER by sensitizing ER Ca^{2+}-release channels and causing protein misfolding. This cycle of Ca^{2+} release, ROS production and protein misfolding act together to activate Ca^{2+}-dependent kinases, calpains, as well as c-Jun *N*-terminal kinase (JNK) or even cell death pathways (Malhotra and Kaufman 2007). The proposed mechanism by which JNK activation occurs, involves the autophosphorilation of IRE-1α that induce conformational change and allows the binding of tumor necrosis factor (TNF)-α-receptor-associated factor 2 (TRAF2) (Urano et al. 2000). Then, IRE-1α-TRAF2 complex recruits JNK, leading to its activation (Davis 2000; Verma and Datta 2012), which consequently will promote caspase-12 cleavage and apoptotic cell death.

3.1.4. Cytokines

Although cytokines have some important roles in OL development, elevated levels of IL-1β, TNF-α and IFN-γ are also correlated with OL toxicity and white matter defects.

IL-1β, which influence pathological processes promoting neurodegeneration and cell death (Farber and Kettenmann 2006), has not evidenced to cause toxicity in OL. As far as we know, the mechanism by which TNF-α causes toxic effects to OL lineage remains unresolved, as well. While some studies found signals of TNF-α toxicity on cultured OL, others did not and some even observed developmental-depend toxicity (Cammer 2000; Jurewicz et al. 2005; Merrill and Scolding 1999; Scurlock and Dawson 1999). However, some authors demonstrated that increased concentrations of TNF-α have the ability to induce OL apoptosis both through the engagement of death receptors, and by activation of sphingomyelinase and release of ceramide (Hovelmeyer et al. 2005; McTigue and Tripathi 2008; Pang et al. 2005; Scurlock and Dawson 1999). Another study has shown that recombinant TNF-α injection into the optic nerve leads to demyelination (Jenkins and Ikeda 1992). In what concerns IFN-γ, the susceptibility of OL to this cytokine is more complex, since it is highly toxic for actively proliferating OPC, much less toxic for immature OL, and not toxic for mature OL (Horiuchi et al. 2006).

Some evidences pointed out that cytokine-induced OL damage may be mediated by iron and involves mitochondrial dysfunction (Zhang et al. 2005). Indeed,

the release of cytokines and free radicals diminish the glutamate uptake due to reduced expression of the glutamate transporters EAAT-2 and EAAT-2, thus resulting in elevated concentrations of this neurotransmitter and consequent overactivation of Ca^{2+}-permeable glutamate receptors that in turns leads to excitotoxicity (Domercq et al. 2007).

3.2. Mechanisms mediated by other central nervous system cells

Toxicity to OL may also be mediated by other cell types in the environment being astrocytes and microglia the most studied ones, due to the production of pro-inflammatory cytokines when they became reactive.

The role of reactive astrocytes and microglia remains unclear, but it is known that they are involved in OL toxicity through the release of highly reactive oxygen/nitrogen species and pro-inflammatory cytokines like TNF-α (Back 2006; Haynes et al. 2005; Li et al. 2008; Rezaie and Dean 2002; Steelman and Li 2011). Moreover, activated microglia present altered glutamate metabolism producing the enzyme glutaminase and the glutamate-cystine exchanger xCT^-, which result in impaired expression or function of glutamate transporters and consequent disruption of glutamate homeostasis and excitotoxicity (Domercq et al. 2007; Matute 2010; Werner et al. 2001). In addition, microglia also express P_{2x7} receptors, which besides having an important role in microglial proliferation and activation (Monif et al. 2009), are also linked to the release of several substances including pro-inflammatory cytokines. Finally, activated microglia may reduce OL survival through the release of peroxynitrite that is toxic to OL (Li et al. 2005).

Despite the fact that astrocytes are generally considered protective by releasing trophic factors such as LIF, some studies are now pointing out a pathological role for reactive astrocytes in white matter diseases. A recent study demonstrated that exogenous levels of TNF-α do not cause significant preoligodendrocyte death in contrast with cultures where these cells are in contact with astrocytes. This data suggest a role for astrocytes in promoting toxicity to OL via TNF receptor 1 activation in a contact-dependent manner (Kim et al. 2011). This contact-dependent toxicity can be dependent on gap junctions since they are known to couple astrocytes and OL (Nagy and Rash 2000; Orthmann-Murphy et al. 2008) and to be involved in the propagation of cell injury (Farahani et al. 2005; Froger et al. 2010; Lin et al. 1998). Moreover, astrocytes were shown to limit remyelination and CNS repair, by forming a glial scar, and to prevent OPC migration and maturation as well as axonal regeneration (Nair et al. 2008).

4. Models to assess changes in oligodendrocyte development and myelination

The different OL maturation stages comprise the new expression of several markers and the loss of other ones, as described before (Fig. I.2). Therefore, to evaluate what is involved in OL development and myelination, or the cross talk between these and the other nerve cells, or their response to toxic or external stimulus, several experimental approaches can be used (Fig. I.8). The ideal features of a cell culture system for *in vitro* investigation depend on what questions the system is to address.

The development of a method to grow OL by mechanical dissociation of OPC from mixed glial cultures (McCarthy and de Vellis 1980) has allowed the evaluation of biological processes and mechanisms of cell death in OPC and their differentiation process into myelinating OL. As described before, in order to maintain these cells in a proliferative stage, the culture media has to include proliferation factors. The mostly used proliferation factors to maintain OPC are PDGF-A and FGF-2, since these factors together allow the maintenance of these cells in continuous proliferation (Bogler et al. 1990). So, in these OPC cultures it can be assayed the mechanisms underlying OPC demise following a certain stimulus. On the other hand, if the proliferating factors are removed from the culture medium and differentiation inducers such as T3 are added OPC will differentiate. In this system, and without the presence of axons, OPC are able to differentiate and synthesize membrane sheets whose organization and composition are very similar to that of myelin (Szuchet et al. 1986), but do not model all aspects of myelination. This culture system allows the evaluation of whether certain molecules may affect OPC differentiation into OL, as well as OL maturation with the characteristic morphological alterations. Indeed, with this model we can categorize the morphology of mature cells in several stages, such as: 1) OL with poorly branched processes; 2) OL with complex branched processes; and 3) cell with complex morphology that partially form membranes. Although in these pure OPC/OL culture systems we can precisely identify the true signalling molecules that are altered in these particular cells when challenged, this type of culture does not allow the reciprocal signalling interactions between OL and neurons which is crucial for the myelination event.

In order to preserve the physiological relevance of neuron-OL interactions and maintain the accessibility of *in vitro* cell culture models, the use of a co-culture system have the advantage of allowing the study of myelination without confounding effects exerted by other cell types. There have been described a few co-culture models using different types of neurons and OL. One of these models comprises dorsal root ganglion

(DRG) neurons, cultured on 3D matrixgel, that are maintained in culture in the presence of NGF and of an anti-mitotic agent (to kill contaminating cells), to which OL (prepared independently) are added following growth factors removal. Among other advantages, DRG neuritis in this system extended radially from their somas facilitating the observation and analysis when compared with co-cultures with neurons dissociated from CNS that are very difficult to analyse due to its reticular distribution (Fex Svenningsen et al. 2003; Thomson et al. 2006). Here, it can be evaluated the effect of biomolecules on the myelination process without the interference of the other glial cells.

In addition, when the purpose of the study is the evaluation of OL development and myelination in conditions very similar to their environment, the use of *ex vivo* organotypic slice cultures should be used instead since the model preserves the tree dimensional architectural organization that supports cell-cell interactions. Slices are thick sections (250-400 μm) that usually are grown on semiporous membranes and kept at an air-liquid interface, which cause less tissue thinning and allow much simple maintenance and processing (Lossi et al. 2009; Stoppini et al. 1991). When evaluation of changes in myelination is the main goal, it is usually used organotypic cerebellar slice cultures, which allows the evaluation of OL maturation process and myelination. This occurs because both OPC and OL are present in cerebellum and compact myelin is formed after some time with a specific pattern (Zhang et al. 2011). Indeed, in cerebellar slice cultures it is possible to follow the complex series of dramatic morphological and biochemical changes that happen during OL maturation and evaluate the effect of biomolecules in each crucial step.

Finally, myelination can be evaluated in animal models. In the last years various animal models have been created in order to evaluate not only changes in normal development and during disease, but also to be used during the screening of therapeutic compounds. Although there are important differences between rodents and humans, the first is one of the most used animal models since analogous structures can be identified. Moreover, the ontogeny of specific behaviors can be applied to draw inferences regarding the maturation of specific brain structures or neural circuits in rodents and humans. As described before, several studies have tried to establish a relationship between the major steps of OL development and myelination in rodents and humans. So it is possible to use animals at different ages in order to mimic specific periods of human development and periods of neurological disorder onset. Moreover, by using *in vivo* animal models it is possible to preserve the brain circuits and evaluate changes in myelination at different brain regions, such as cerebellum, brainstem, medulla oblongata, pons, corpus callosum and hippocampus. Using this type of

approach it is also possible to evaluate changes in the systemic circulation and their effects in the brain, being very important for the study of diseases that are initiated at the systemic level and have a secondary CNS phenotype or CNS disorders that will progress and exacerbate following immune cell infiltration.

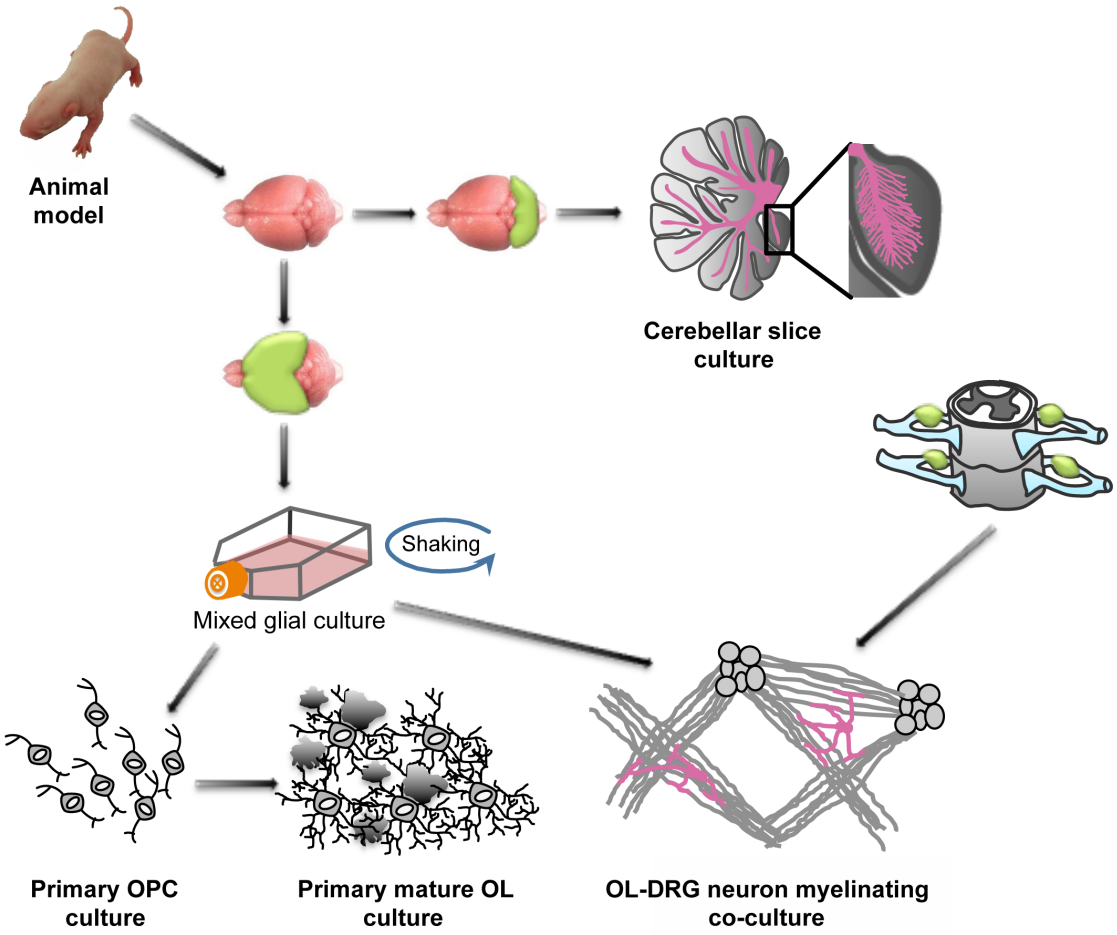


Fig. I.8 – Models to evaluate oligodendrocyte (OL) development and myelination. OL are obtained from dissociated cortexes plated on flasks in order to obtain mixed glial cultures. After dissociation, by shaking, OL are plated out on coverslips with media supplemented with proliferation factors in order to obtain primary oligodendrocyte precursor cell (OPC) culture or in media without these factors to allow OL maturation and to obtain primary mature OL cultures. To evaluate changes in myelination, two *in vitro* models can be used, as follows: OL-dorsal root ganglia (DRG) neurons myelinating co-culture or cerebellar slice cultures. In myelinating co-cultures OL are obtained by the same method, added to DRG neurons from embryonic spinal cord and cells are maintained in culture to allow myelination. Organotypic cerebellar slice cultures are obtained from rodent brain and cultured on membranes to study myelination. Finally, several animal models are available to evaluate changes in myelination in several brain regions.

5. Oligodendrocyte involvement in some neurological disorders

Since OL and the consequent ability to myelinate neuronal axons are so important for the fast conduction of the action potential and the maintenance of the axonal integrity, their pathophysiology is emerging as a key event in the occurrence of neurological disorders. Defects in myelin insulation can lead to several CNS disorders along life journey. Indeed, impairment of OPC and OL function may occur during the perinatal life, in conditions such as neonatal hyperbilirubinemia and trigger the emergence of neurological conditions, such as schizophrenia. On the other hand, OL damage and myelin loss may also be the genesis of several neurodegenerative diseases including multiple sclerosis.

5.1. Schizophrenia

Schizophrenia is considered a severe psychiatric disorder because of its chronic course and often poor long-term outcomes in social and vocational realms (Henry et al. 2010). In addition, schizophrenia is the most common psychotic illness, with approximately 7 in 1000 people developing the disorder in their lifetime (McGrath et al. 2008). This condition is characterized by: 1) positive symptoms, such as delusions, hallucinations and disorganized speech/thinking; 2) negative symptoms, like social withdrawal, anhedonia and blunted affect; and 3) cognitive dysfunction, including deficits in attention, working memory and executive function (American Psychiatric Association 2011; Foussias and Remington 2010).

The exact cause for schizophrenia is still unknown. However, their association with previous exposure to prenatal infection was demonstrated (Brown 2006), especially because cytokines have an important role in neurite impairment (Grima et al. 2003), which is in accordance with the neuropathology of this disease. In addition, the incidence of schizophrenia is increased in patients that present Gilbert syndrome or that had hyperbilirubinemia during the neonatal period (Dalman and Cullberg 1999; Miyaoka et al. 2000). More recently, it is hypothesized that the etiology of schizophrenia is the result of both abnormalities in local activity within various brain regions and dysfunctional interactions between cortical and subcortical circuits (Swerdlow 2010), probably due to abnormalities in brain development during fetal/neonatal life long before manifestation of illness in adolescence or early adulthood (Fatemi and Folsom 2009).

Concerning to changes in neuronal circuits, impairment in synapse formation and plasticity has been implicated in schizophrenia (Daskalakis et al. 2008; Harrison and Weinberger 2005; Stephan et al. 2006; Stephan et al. 2009). In addition, it is

accepted that dopaminergic activity can modulate symptoms of schizophrenia, although the degree to which dopaminergic activity is a primary or secondary consequence of the disease is still unsolved. However, some studies identified neuroanatomical changes in prefrontal cortex due to loss of glutamatergic pyramidal cell spines and axons, loss of GAGAergic interneurons and decreased mesocortical dopaminergic innervation, while others attributed cognitive impairments to cell loss within thalamic subregions and subsequent decrease in excitatory thalamic afferents to the prefrontal cortex (Lewis and Sweet 2009; Smith et al. 2011; Volk and Lewis 2010). Also been reported are alterations in glutamatergic, GABAergic and dopaminergic signalling, leading to loss of neuronal connections or neurons in other brain regions, like hippocampus, striatum, amygdala and auditory and visual cortex (Benes 2009; Dorph-Petersen et al. 2009; Dorph-Petersen et al. 2007; Simpson et al. 2010; Smiley et al. 2011). Moreover, this disorder leads to an increase in dopamine release in the striatum in parallel to its depletion in prefrontal cortex (Featherstone et al. 2007). Another important feature observed in this condition is a decreased NMDA receptor function in subcortical regions, disinhibiting glutamate and dopaminergic signalling in the cortex, with consequent sensory, cognitive and behavioral deficits (Lisman et al. 2008; Moghaddam and Adams 1998; Olney et al. 1999).

Although most studies on schizophrenia brain defects have focused on alterations in neurons and gray matter, more recent reports also implicate white matter damage underlying the neurodevelopmental alterations. Indeed, given the clear impact that changes in glutamate have on neuronal plasticity and synaptic connectivity, it can be postulated that it may also compromise the integrity of the white matter by acting directly on OL. Moreover, disorganized thought and cognitive impairments observed in schizophrenia can be related with altered conduction velocity (Thaker 2008), since defined conduction velocity is necessary for several learning processes (Fields 2008). In fact, the expression levels of Olig2, MBP, MOG and MAG are lower in dorsolateral prefrontral cortex of patients with schizophrenia when compared with control subjects (Hakak et al. 2001; Tkachev et al. 2003). Furthermore, many studies demonstrated impairment in white matter integrity and organization in several brain regions, including prefrontal, temporal and occipital lobes (Heimer 2000; Kanaan et al. 2005; Kubicki et al. 2007; Kubicki et al. 2005), as well as reduced white matter tracts connecting corticothalamic and cortolimbic structures, evidencing the disconnection of these networks (Ellison-Wright and Bullmore 2010). The possible explanation for the decreased white matter and the down expression of myelin-related genes in this disorder is that OL are either present in reduced number or are dysfunctional. Interestingly, some authors have already shown that patients with schizophrenia

present a more disperse arrangement of OL and lower density of OL in gray matter, in white matter in the superior frontal gyrus (Hof et al. 2003), in anterior cingulate cortex (Stark et al. 2004) and in anterior thalamic nucleus (Byne et al. 2008). By electron microscopy it is possible to observe damaged myelin sheath lamellae forming lamellar bodies in schizophrenic brains, as well as irregularities in OL mitochondria and their apoptosis (Uranova et al. 2001). Finally, ultrastructural studies showed OL loss in the fascicular white matter, with shrinkage in the diameter of neuronal axons (Uranova et al. 2007), confirming myelination alterations in schizophrenic patients.

Although it is clear that there is not a single locus of dysfunction within the schizophrenic brain, several questions remain to be elucidated, principally the identification of factors leading to OL and neuronal dysfunction and the most affected cells.

5.2. Multiple sclerosis

Multiple sclerosis (MS) that is the most common neurological disease between young adults (with ages between 20 and 40 years) has a worldwide prevalence estimated between 1.1 and 2.5 million cases (Liguori et al. 2000; Pugliatti et al. 2002). MS is a very complex disease, with variable onset and clinical course that involve several pathophysiological mechanisms, including axonal/neuronal damage, demyelination, inflammation, gliosis, oxidative stress and excitotoxicity, followed by remyelination and repair, together with immune system alterations and BBB disruption (Bielekova and Martin 2004; Pugliatti et al. 2002). The onset of the disease is characterized by episodes or relapses of common symptoms, like impaired vision and deficits in sensation (Baranzini 2009). At later stages patients can present severe disabilities including paralysis, incontinence and cognitive impairment, involving memory loss and concentration problems (Al-Omaishi et al. 1999; Baranzini et al. 2009). Full recovery from initial relapses usually occurs during the early stages of the disease course, but after a variable period of relapsing-remitting phase (RRMS) a progressive build-up of residual neurological dysfunction develops along the secondary progressive phase (SPMS). However, approximately 10 to 20% of the individuals with MS show a progressive disease since the onset with no relapse or remission episodes, termed primary progressive MS (PPMS).

RRMS is dominated by multifocal inflammation and cytokine physiological actions (Bielekova and Martin 2004) through the gradual accumulation of these biomolecules at this phase and consequent irreversible neurological deficits, leading to

SPMS that is characterized by clinical attacks and remissions, with progression of the clinical symptoms (Arnett 2010; Ontaneda and Rae-Grant 2009; Rejdak et al. 2010).

PPMS is generally characterized by a lesser degree of inflammation and a greater proportion of axonal loss, even during the early disease course (Bashir and Whitaker 1999).

Altough the etiology of MS remains elusive, plaques of inflammatory demyelination within the CNS are considered the pathologic hallmark of MS, being destructed myelin an essential element within these plaques (Compston 2006; Franciotta et al. 2008). Some studies also reveal that BBB is a key structure (Compston and Coles 2002; Compston and Coles 2008), since the entry of cells from the immune system into the CNS is a critical step for the onset of the disease especially during the acute phases. Relapses are fundamentally a manifestation of an inflammatory response occurring mostly in the white matter, but also in the myelin tracts of the grey matter resulting in focal demyelination and relative axonal sparing. During the past years, MS research has mostly focused on the role of $CD4^+$ T cells in the disease pathogenesis. Immune phase begins with $CD4^+$ T cells activation in response to dendritic cells that take up the exogenous or endogenous antigen in order to present it to the immune cells. As a result, $CD4^+$ T cells become activated and secrete IFN-γ, TNF-α, TGF-β, IL-10 and IL-17 (Wootla et al. 2012). Moreover, T cells from MS patients can recognize MBP (Pette et al. 1990; Valli et al. 1993), PLP (Greer et al. 1997) and MOG (Zhang et al. 1994). These cells have also the ability to stimulate microglia, macrophages and astrocytes and to recruit B cells, ultimately resulting in demyelination and damage of OL and axons with concomitant neurological deficits (Hohlfeld 2010). Moreover, B cells may directly participate in the demyelination process by secreting pathogenic antibodies that target OL with or without the presence of complement (O'Connor et al. 2005).

Although demyelination has long been considered a marked feature of MS in proportion to the loss of axons, axonal damage is an important finding in this disease that correlates with its progression and permanent neurologic disability in patients (Ferguson et al. 1997). In fact, some studies proposed that axonal impairment occurs in areas of active inflammatory demyelination and in an early phase of the disease course (Kuhlmann et al. 2002; Trapp et al. 1998). This axonal loss contribute to the clinical decline observed in MS patients, since a reduced number of surviving corticospinal axons are correlated with high levels of motor disability (Tallantyre et al. 2010). The exact mechanism by which axonal damage arises is not completely solved. However, axonal damage can be induced by $CD8^+$ T cells via the release of cytotoxic granules, induction of apoptosis through activation of surface receptors, the release of

cytokines from surrounding glia or immune cells, or direct transection of axons (Medana et al. 2001; Neumann et al. 2002). Moreover, microglia can also release toxic molecules such as proteases and nitrogen species leading not only to axonal degeneration but also to OL injury, demyelination and BBB dysfunction (Smith and Lassmann 2002). As a consequence of the immune injury to myelin, glutamate-mediated toxicity and higher energy demands may further increase the damage (Mahad et al. 2009).

5.3. Neonatal hyperbilirubinemia

As described above changes in white matter observed in some diseases can be due to changes in OL viability or myelination during the early neonatal life. In this context, hyperbilirubinemia or jaundice is a frequent condition during this period that affects 60% of full-term newborns and 80% of preterm infants (Bhutani et al. 2004; Maisels and McDonagh 2008; Vreman et al. 2004). The current understanding on the development of bilirubin encephalopathy is that when the level of serum unconjugated bilirubin (UCB) exceeds the bilirubin binding capacity of albumin, occurs an increase in the amount of unconjugated unbound UCB (free bilirubin), therefore increasing its passage across the BBB and saturating the brain cellular defensive mechanism. In these conditions, either UCB uniformly distributes in the brain parenchyma (bilirubin-induced brain dysfunction or bilirubin-induced neurological dysfunction (BIND)) or specifically precipitates in some areas such as basal ganglia, central and peripheral auditory pathways, hippocampus, diencephalon, subthalamic nuclei and cerebellum (*kernicterus* or bilirubin encephalopathy), thus resulting in lesions that may be reversible or not, depending on duration, developmental age, and concomitant pathologies (Bhutani and Stevenson 2011; Brites 2012).

UCB is formed by the catabolism of heme, present in erythrocyte hemoglobin, (Gourley 1997; Stevenson et al. 2001). Given the particular physiology of newborns and preterm infants in relation to bilirubin metabolism, it is commonly observed an increase in total serum bilirubin (TSB). Regarding bilirubin production, newborns produce more than twice of the amount produced by adults (Bartoletti et al. 1979; Maisels et al. 1971), due to a higher number of circulating red blood cells per kilogram and a shorter erythrocyte lifespan (Blackburn 2003; Maden et al. 2005; Wong et al. 2006). In addition, plasma albumin levels, the bilirubin carrier in systemic circulation, are lower in newborns and correlated with gestational week (Notarianni 1990). Moreover, newborns have to provide the clearance of systemic UCB excretion that was performed by placenta in the intrauterine life. This excretion occurs mainly through

conjugation with one or two molecules of glucuronic acid at the liver, by using the enzyme uridine 5'-diphospho-glucuronosyl transferase (UGT), which is encoded by UGT1A1 gene. In this regard, in newborns, during the first 10 days after birth, UGT1A1 gene activity is less than 1% that of adult values. Therefore, neonates evidence a decreased ability to conjugate and excrete UCB (Kawade and Onishi 1981; Onishi et al. 1979). Another mechanism that contributes to newborn jaundice is the increased enterohepatic circulation of bilirubin. To that it may account an increased presence of β-glucuronidase in the intestine, thus accomplishing a higher deconjugation of UCB than usual, the absence of intestinal flora to perform the transformation of UCB in compounds that are excreted in stools, and the decreased intestinal motility. Concerning the β-glucuronidase its levels are reported to be higher in the newborn's intestine than in adults (Gartner 1994), and in the breastfeeding babies (Gourley 2002). In addition the low intestinal flora decreases the transformation of UCB into urobilinogen and urobilins to be excreted by faeces and urine, promoting its absorption and its increase in circulation (Gartner 1994; Maisels 2005). Finally, a decreased intestinal motility favors the bilirubin mono- and digluronide deconjugation by β-glucuronidase triggering its absorption by the intestinal mucosa (Boyer and Vidyasagar 1987), thus also increasing the UCB recirculation.

Most cases of jaundice in newborns, defined as "physiologic jaundice" (Ostrow et al. 2002), occur in a transient and non-injurious manner due to normal maturational limitations of newborns. This term is applied to newborns whose TSB is within the normal range for the first week of life, but the problem is to define what it may be considered this range, since this value varies between populations, and several studies report different values and limits. However, recent data suggest a higher upper limit of normal TSB in the modern population, varying from 14 mg/dL (239 μmol/L) to 17 mg/dL (291 μmol/L) (Johnson and Bhutani 2011; Maisels and Kring 2006). In contrast moderate jaundice is applied to values between 17 mg/dL and 20 mg/dL (342 μmol/L) and severe jaundice to levels between 20 mg/dL and 25 mg/dL (427 μmol/L). Newborns with values of TSB above 25 mg/dL are considered at risk of developing chronic bilirubin encephalopathy, referred as *kernicterus*, a condition usually leading to chronic and permanent clinical *sequelae* or death (Johnson and Bhutani 2011). Nevertheless, these values may not be viewed as absolute ones, since the same UCB concentrations may reveal safe in normal newborn infants while become potentially hazardous in premature infants (Stevenson et al. 2001; Watchko and Maisels 2003) or even in term neonates displaying sepsis (Hansen et al. 1993; Kaplan and Hammerman 2005) or hypoxia-ischemia (Ahdab-Barmada and Moossy 1984; Falcão et al. 2007b), which have been identified as risk factors for bilirubin encephalopathy. Regarding

prematurity, data from *in vitro* (Falcão et al. 2005; Falcão et al. 2006) and *in vivo* studies (Conlee and Shapiro 1997; Keino and Kashiwamata 1989) point to the existence of windows of developmental susceptibility of the CNS to bilirubin toxicity (Shapiro 2010). In this context, these findings suggest that immature neurons undergoing differentiation at the time of bilirubin exposure are more susceptible to cell death, while slightly mature cells may show transient changes. In fact, premature infants have a greater risk for developing *kernicterus* than full-term infants and this condition can occur for lower levels of TSB (Maisels and Watchko 2003; Watchko and Maisels 2003; Watchko and Oski 1992).

Symptomatically, neonatal jaundice is characterized by a yellowish coloration that progresses in a cephalocaudal direction. The face and sclera typically appear icteric when TSB reaches 6 to 8 mg/dL, whereas the entire body, including palms and soles, become yellow at values of 12 to 13 mg/dL (Hatzenbuehler et al. 2010). Adverse consequences of severe jaundice include from reversible manifestations, such as lethargy and decreased feeding, hypotonia or hypertonia, high-pitched cry, fever and seizures (Shapiro 2003), to irreversible *sequelae* characterized by the classical manifestations of choreoathetoid cerebral palsy, isolated auditory loss and dental enamel hypoplasia that may culminate in death (Dennery et al 2001; American Academy of Pediatrics 2004; Bhutani 2010). Thus, BIND encompasses these more subtle neurological impairments (Shapiro 2005), including mild neurological abnormalities (Hyman et al. 1969; Naeye 1978; Rubin et al. 1979) and isolated neural hearing loss (Bergman et al. 1985; Salamy et al. 1989). In addition, concerns about subtle manifestations of BIND, due to levels of hyperbilirubinemia that are not generally considered severe enough to indicate treatment, or to prolonged exposure to lower levels of bilirubin in a vulnerable infant, have already been validated in previous studies (Bhutani 2001; Fernandes et al. 2009; Shapiro 2005; Soorani-Lunsing et al. 2001). A very recent study also demonstrated that bilirubin levels falling short of developing acute bilirubin encephalopathy affects neurodevelopmental outcome, with a proportional increase in the abnormal developmental quotient and peak of serum bilirubin (Arun Babu et al. 2012). These results corroborate previous studies, which demonstrated that neonatal jaundice could have an impact in learning and memory and changes in long-term cognitive ability (Seidman et al. 1991; Zhang et al. 2003). In fact, an increased risk of psychological development disorders, especially autistic disorders, was observed for children exposed to jaundice as neonates (Gardener et al. 2011; Maimburg et al. 2010; May-Benson et al. 2009). Moreover, as described above, schizophrenia has also a higher incidence in patients that developed neonatal jaundice or patients with Gilbert syndrome when compared with patients without jaundice

(Dalman and Cullberg 1999; Miyaoka et al. 2000). Gilbert syndrome is a condition most commonly diagnosed in young adults that affect 6% to 9% of the population (Maisels 2005). This condition is due to a mutation in the UGT1A1 gene promoter or missense mutations in the coding region that reduce UGT activity (Maisels 2005). Consequently, these individuals present slightly increased levels of UCB (<5 mg/dL) along life (Watchko and Lin 2010).

Histological *post-mortem* findings show that bilirubin affect nerve cells. Indeed, brain sections of jaundiced infants showed UCB within neurons, neuronal processes and microglia (Hansen 2000; Martich-Kriss et al. 1995).

5.3.1. Overview to the mechanisms underlying bilirubin-induced toxicity

UCB accumulation in the brain can occur due to changes in the expression of two ATP-dependent plasma membrane efflux pumps, multidrug resistance-associated protein 1 (Mrp1) and P-glycoprotein (Pgp), that are described as to be responsible for limiting UCB passage across the BBB and its intracellular accumulation (Bellarosa et al. 2009; Daood et al. 2009; Sequeira et al. 2007). In this context, some studies revealed that Pgp expression is decreased in early-life (Daood et al. 2009; Gazzin et al. 2011), while Mrp1 is down regulated in situations of prolonged exposure to elevated concentrations of UCB (Gazzin et al. 2011). Moreover, UCB passage through the BBB can also be favored due to a compromised integrity of the BBB during increased UCB in circulation. Indeed, it was observed that UCB promotes NO production in human brain microvascular endothelial cells (HBMEC) (Palmela et al. 2011), a biomolecule described as an endothelial-derived relaxing factor (Bellefontaine et al. 2011) with the ability to increase microvascular permeability (Kim and Jung 2011). Interestingly, recent studies from our group evidenced that prolonged exposure of HBMEC to high concentrations of UCB impairs barrier function, with an increase in paracellular permeability and a decrease in transendothelial electrical resistance of the monolayer (Palmela et al. 2012) (Fig. I.9).

Once in the brain, bilirubin-induced neurotoxicity occurs first at the cell membrane, causing changes in the polarity, fluidity, protein order and redox status (Brito et al. 2001; Rodrigues et al. 2002b). UCB is also able to interact with the mitochondrial membrane triggering mitochondrial swelling, Bax translocation and cytochrome *c* release in neurons, with consequent caspase-3 activation, PARP cleavage and apoptotic cell death (Rodrigues et al. 2002a,b). Later on, UCB-induced neuronal apoptosis was shown to involve both pathways of apoptosis, intrinsic and extrinsic, with activation of caspase-8, -9 and -3, following NO production and JNK 1/2 activation (Vaz et al. 2011). Apoptosis in neurons exposed to UCB involve the

activation of NMDA receptors as a consequence of excitotoxicity (Brito et al. 2010; Grojean et al. 2000; McDonald et al. 1998). The neuron susceptibility to death induced by UCB depends on their maturation stage since immature neurons are more vulnerable than the most differentiated ones (Falcão et al. 2006), probably due to a reduced expression of Mrp1 (Falcão et al. 2007a). In addition, UCB inhibits cytochrome *c* oxidase activity in immature neurons, which is accompanied by bioenergetic and oxidative crisis, increased glycolytic activity, superoxide anion radical production, ATP release and disruption of glutathione redox status (Vaz et al. 2010). Moreover, early exposure of developing neurons to UCB lead to neuritic atrophy, reduced neuronal arborization, arrested neuritic growth and neuritic hypoplasia (Fernandes et al. 2009; Shapiro 2005). UCB also triggers synaptotoxicity, through reduction of dendritic spines and synapse densities (Fernandes et al. 2009), as well as decrease in synaptophysin and synaptosomal-associated protein 25 expression (Silva et al. 2012). These proteins are involved in synapse establishment and neurotransmitter release (Wang and Tang 2006), and as so, the effect of UCB has later been related to potentiation of inhibitory synaptic transmission (Shi et al. 2006) and impairment of the long-term potentiation and long-term depression (Chang et al. 2009).

Regarding glial cells, it was also demonstrated that UCB triggers cell death in astrocytes, microglia and OL (Fernandes et al. 2004; Genc et al. 2003; Gordo et al. 2006), being astrocytes the less vulnerable cells and microglia the most susceptible ones (Brites et al. 2009; Falcao et al. 2006; Silva et al. 2002). As observed for neurons UCB leads to a fast increase in extracellular glutamate (Fernandes et al. 2004), mainly in immature astrocytes (Falcão et al. 2005), that appears to result from an increase in astrocyte secretion and an inhibition of its uptake (Falcão et al. 2006; Silva et al. 1999). Increase in extracellular glutamate is an important factor of UCB neurotoxicity, since the prolonged presence of glutamate in the synaptic cleft can overstimulate NMDA receptors leading to excitotoxicity (Grojean et al. 2001; McDonald et al. 1998). Both, astrocytes and microglia, present an inflammatory response after exposure to UCB, causing the release of pro-inflammatory cytokines, such as TNF-α and IL-1β (Fernandes et al. 2006; Fernandes et al. 2004; Gordo et al. 2006; Silva et al. 2011). In astrocytes the effect of UCB includes upregulation of receptors for TNF-α and IL-1β (Fernandes et al. 2011), activation of mitogen-protein kinases (MAPKs) p38, JNK 1/2, extracellular signal-regulated kinase (ERK) 1/2, and nuclear factor-kappaB (NF-κB) signalling pathway (Fernandes et al. 2007; Fernandes et al. 2006). Similarly to astrocytes, UCB activates MAPKs and NF-κB in microglia, but these cells, respond much earlier than astrocytes, as expected (Silva et al. 2010). In addition, UCB has the ability to increase microglia phagocytic ability (Silva et al. 2011). Interestingly, in the

presence of neurons UCB-induced microglia phagocytosis was increased possibly to remove neuronal debries (Silva et al. 2012). In addition, using an organotypic hippocampal slice system where microglia was depleted by using clodronate lipossomes, we showed that microglia are responsible, at least in part, for the UCB-induced release of glutamate and NO, as well as for consequent neuritic impairment and cell death (Silva et al. 2012).

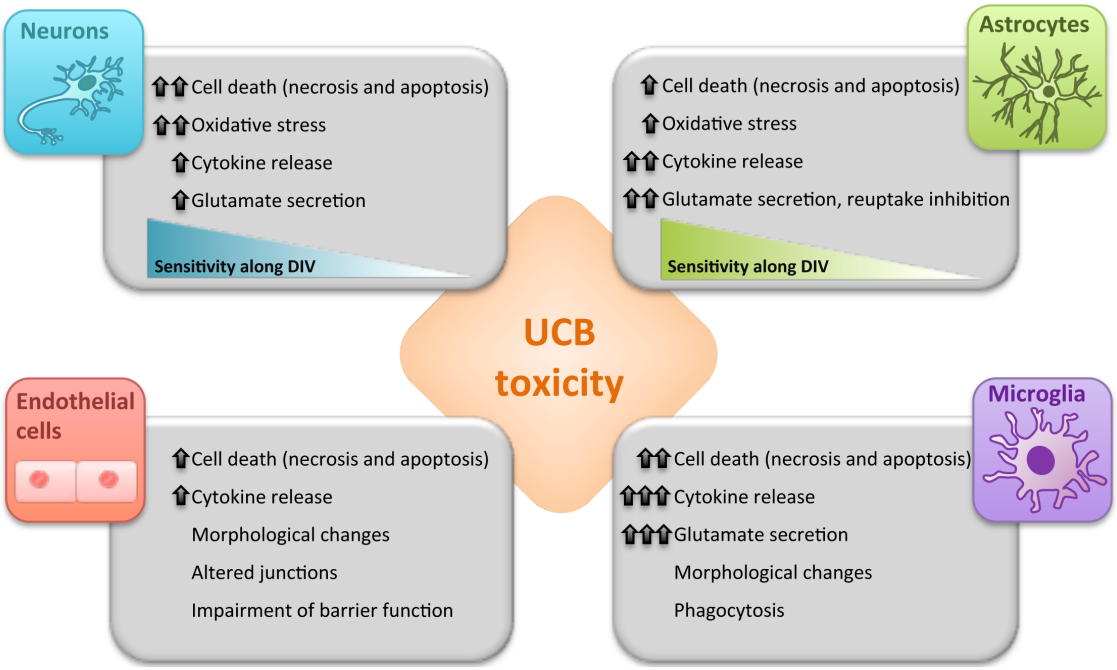


Fig. I.9 – Major findings regarding the molecular mechanisms on cell function induced by unconjugated bilirubin (UCB). UCB leads to cell death by necrosis and apoptosis, an inflammatory response in neurons, astrocytes, microglia and endothelial cells. In addition, UCB induces oxidative stress and glutamate release in neurons and astrocytes, morphological changes and phagocytosis in microglia and altered junctions and impairment of barrier function in endothelial cells from the blood brain barrier. Moreover, UCB toxicity to neurons and astrocytes is depending on their maturation stage, since immature cells are more vulnerable than mature ones. Data were reviewed in Brites 2012. DIV, *days in vitro*.

5.3.2. Does bilirubin compromise myelination?

Concerning the UCB effects on OL, only one study has previously demonstrated that high levels of UCB decrease the viability of mature OL, through the induction of NO production (Genc et al. 2003). However, there are some evidences that UCB can lead to changes in white matter. In this context, recent results have demonstrated that myelination is altered in a premature infant with *kernicterus*, showing a decrease in the density of myelinated fibers and loss of axons in the cerebellum (Brito et al. 2012). These findings are in line with previous studies that have already evidenced that cerebellum is the most common pigmented region of the brain, after basal ganglia, with a marked decrease in the number of neurons, an increase in

gliosis and a reduction in the myelination pattern (Ahdab-Barmada and Moossy 1984; Claireaux 2003; Silberberg and Schutta 1967). Moreover, white matter volume reduction and delay in hemispheric myelination was also observed in infants with severe UCB encephalopathy outcomes (Gkoltsiou et al. 2008). To this regard, it was already demonstrated that UCB is able to bind mainly to MBP and that high concentration of this pigment is found in the myelin fraction of rat brains injected with UCB (Gurba and Zand 1974; Hansen et al. 2001) (Fig. I.10). In more detail, an early study performed in an experimental model of *kernicterus* demonstrated that this condition lead to significant changes in myelin sheath, with separation of myelin lamellae, suggesting that its compactation did not properly occured (Chen et al. 1971) (Fig. I.11). Interestingly, another study in Gunn rats, an animal model of *kernicterus,* showed by electronic microscopy that myelin figures present tongues or remmants of cytoplasm and irregular spaces or vacuoles. This study also revealed the presence of myelin around vacuoles, bits of cytoplasm and other cytoplasmic debris (Jew et al. 1977).

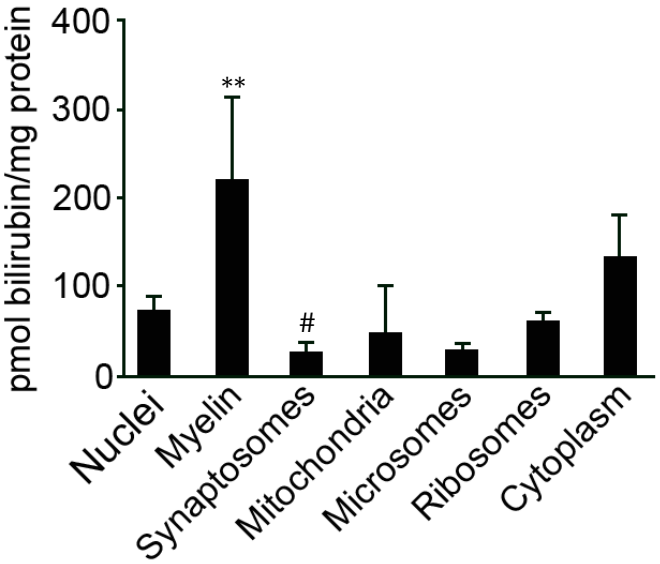


Fig. I.10 – Bilirubin subcellular localization in the rat brain 10 min after an I.V. administration of a bilirubin bolus of 50 mg/kg over a period of 5 min. Bilirubin was shown to bind mainly to myelin, when compared with other brain subcellular organelles, such as nuclei, synaptosomes, mitochondria, microsomes, ribosomes and cytoplasm. **P*<0.01 vs. microsomes, synaptosomes and mitochondria; #*P*<0.05 vs. ribosomes. Adapted from Hansen et al. 2001.

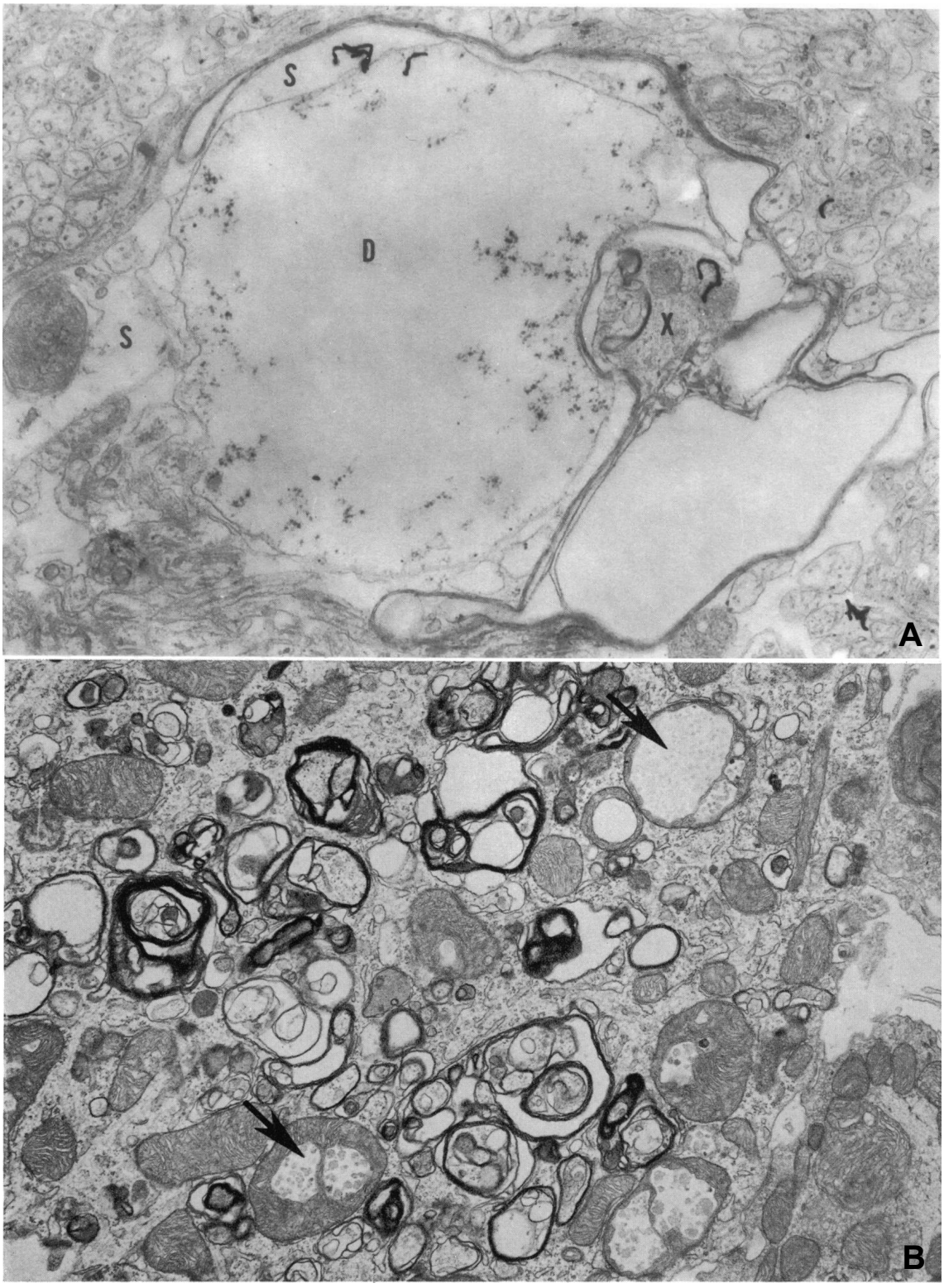


Fig. I.11 – Ultrastructural abnormalities observed in asphyxiated rabbits with less than 24 hours old after an intravenous injection of bilirubin (a) and in the hyperbilirubinemic Gunn rat model (aged 4 weeks) (b). a Dendrite with dense granules and splitting of myelin lamellae in axon by electron microscopic autoradiograph (x16,000). D, dendrite; S, astrocyte process; X, axon. From Chen et al. 1971. **b** Representative image of myelin figures surrounding vacuoles, bits of cytoplasm, and debris, mitochondria swelling, vacuolation and glycogen accumulation (arrows) obtained by electron microscopy (x13,600) . From Jew et al. 1977.

All together, these results suggest that UCB may affect myelination, but there are no evidences about the possible mechanisms affecting this process and the development of OL. Since hyperbilirubinemia can determine lasting *sequelae* and it is speculated that it may favors the appearance of other neurological diseases and even increased susceptibility to ageing, it will be very important to understand how this condition is really affecting the OL viability and maturation, as well as the myelination process.

6. Global aims of the thesis

The main goal of the present work was to explore if hyperbilirubinemia has the ability to induce changes in OL development and myelination.

As a first step we decided to investigate whether UCB promoted OPC demise and which molecular mechanisms were involved, taken in consideration that all prior data evidenced bilirubin-induced damage to neurons, astrocytes and OL and because OPC are indicated as the most vulnerable cells of OL lineage. Therefore, we evaluated the effects of UCB in the survival of OPC and the pathways involved in UCB-induced OPC demise, including intracellular mechanisms such as: 1) ER stress, 2) mitochondrial dysfunction, 3) ROS production, cytokine and glutamate release, 4) JNK activation, and 5) calpain activation.

In a second step, and hypothesizing that OPC should be highly vulnerable to UCB exposure through the activation of several mechanisms of cell death, we assumed that UCB would alter OL maturation and their ability to myelinate. So, we planned to first evaluate the UCB effects on OL maturation using an *in vitro* model of OL differentiation and in line with this we determined: 1) the maturation stage of OL; 2) the alteration in OL developmental transcription factors; 3) the changes produced in OL morphological maturity; and 4) the cytoskeletal-related mechanisms responsible for alterations in the morphology.

Then, we programmed to evaluate the ability of UCB exposure to induce changes in the myelination pattern, using a myelinating co-culture model (e.g. OL-DRG neurons co-culture). In this context, the myelinating-related parameters that we considered to be evaluated after UCB exposure were: 1) the differentiation stage of OL; 2) their morphological maturation prior to myelination; 3) the number of myelinated internodes by each OL; and 4) the internode length.

Considering that UCB would impair OL maturation and myelination in myelinating co-cultures, we established to evaluate UCB effects in a more complex model of myelination, the organotypic cerebellar slice cultures. This system not only allows the study of myelination in a tree dimensional architectural organization, but also permits the influence of other glial cells in UCB-induced response, namely the role of other important risk factors during the perinatal period, like pro-inflammatory cytokines. At this point determinations related with both myelination and gliosis features were

selected as follows: 1) the maturation stage of OL; 2) the percentage of myelinated fibers; 3) the changes in microgliosis and astrocytosis; and 4) the release of molecular OL toxicants, such as glutamate and cytokines.

Finally, and in an attempt to be closer to the alterations produced by BIND in the neonatal life we thought to investigate the defects in myelination and glia reactivity in a promising, and still not published, newborn mouse animal model of severe hyperbilirubinemia or *kernicterus* by assessing 1) brain morphological changes; 2) myelinating alterations; and 3) microgliosis and astrocytosis features in the cerebellum, medulla oblongata, pons and corpus callosum, having a special consideration by the first brain region indicated, one of the most affected by the condition.

All together, we expect that the established experimental approaches here indicated will contribute to elucidate the mechanisms, at least some of them, that mediate the impaired neurodevelopmental myelinogenesis in BIND and kernicteric conditions, in order to define targets to be explored in the assessment of novel neuroprotective strategies. Indeed, we believe that neuroprotective therapies cannot be fully successful without oligoprotection.

7. References

Ahdab-Barmada M, Moossy J. 1984. The neuropathology of kernicterus in the premature neonate: diagnostic problems. Journal of neuropathology and experimental neurology 43(1):45-56.

Al-Omaishi J, Bashir R, Gendelman HE. 1999. The cellular immunology of multiple sclerosis. Journal of leukocyte biology 65(4):444-52.

Allen NJ, Barres BA. 2009. Neuroscience: Glia - more than just brain glue. Nature 457(7230):675-7.

Alliot F, Godin I, Pessac B. 1999. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Brain research Developmental brain research 117(2):145-52.

Almazan G, Honegger P, Matthieu JM. 1985. Triiodothyronine stimulation of oligodendroglial differentiation and myelination. A developmental study. Developmental neuroscience 7(1):45-54.

Amano M, Fukata Y, Kaibuchi K. 2000. Regulation and functions of Rho-associated kinase. Experimental cell research 261(1):44-51.

American Academy of Pediatrics: Clinical Practice Guideline: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. 2004. Pediatrics 114:297-316.

American Psychiatric Association. Proposed draft revisions to DSM disorders and criteria. 2011.

Angulo MC, Kozlov AS, Charpak S, Audinat E. 2004. Glutamate released from glial cells synchronizes neuronal activity in the hippocampus. The Journal of neuroscience: the official journal of the Society for Neuroscience 24(31):6920-7.

Araque A, Navarrete M. 2010. Glial cells in neuronal network function. Philosophical transactions of the Royal Society of London Series B, Biological sciences 365(1551):2375-81.

Armstrong RC, Dorn HH, Kufta CV, Friedman E, Dubois-Dalcq ME. 1992. Pre-oligodendrocytes from adult human CNS. The Journal of neuroscience: the official journal of the Society for Neuroscience 12(4):1538-47.

Arnett HA, Fancy SP, Alberta JA, Zhao C, Plant SR, Kaing S, Raine CS, Rowitch DH, Franklin RJ, Stiles CD. 2004. bHLH transcription factor Olig1 is required to repair demyelinated lesions in the CNS. Science 306(5704):2111-5.

Arnett PA. 2010. Does cognitive reserve apply to multiple sclerosis? Neurology 74(24):1934-5.

Artal-Sanz M, Samara C, Syntichaki P, Tavernarakis N. 2006. Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. The Journal of cell biology 173(2):231-9.

Artal-Sanz M, Tavernarakis N. 2005. Proteolytic mechanisms in necrotic cell death and neurodegeneration. FEBS letters 579(15):3287-96.

Arun Babu T, Bhat BV, Joseph NM. 2012. Association between peak serum bilirubin and neurodevelopmental outcomes in term babies with hyperbilirubinemia. Indian journal of pediatrics 79(2):202-6.

Baas D, Bourbeau D, Sarlieve LL, Ittel ME, Dussault JH, Puymirat J. 1997. Oligodendrocyte maturation and progenitor cell proliferation are independently regulated by thyroid hormone. Glia 19(4):324-32.

Baas D, Legrand C, Samarut J, Flamant F. 2002. Persistence of oligodendrocyte precursor cells and altered myelination in optic nerve associated to retina degeneration in mice devoid of all thyroid hormone receptors. Proceedings of the National Academy of Sciences of the United States of America 99(5):2907-11.

Back SA. 2006. Perinatal white matter injury: the changing spectrum of pathology and emerging insights into pathogenetic mechanisms. Mental retardation and developmental disabilities research reviews 12(2):129-40.

Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC. 2001. Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. The Journal of neuroscience: the official journal of the Society for Neuroscience 21(4):1302-12.

Bacon C, Lakics V, Machesky L, Rumsby M. 2007. N-WASP regulates extension of filopodia and processes by oligodendrocyte progenitors, oligodendrocytes, and Schwann cells-implications for axon ensheathment at myelination. Glia 55(8):844-58.

Baerwald KD, Popko B. 1998. Developing and mature oligodendrocytes respond differently to the immune cytokine interferon-gamma. Journal of neuroscience research 52(2):230-9.

Bakiri Y, Burzomato V, Frugier G, Hamilton NB, Káradóttir R, Attwell D. 2009. Glutamatergic signaling in the brain's white matter. Neuroscience 158(1):266-74.

Bansal R, Pfeiffer SE. 1994. Inhibition of protein and lipid sulfation in oligodendrocytes blocks biological responses to FGF-2 and retards cytoarchitectural maturation, but not developmental lineage progression. Developmental biology 162(2):511-24.

Baranzini SE. 2009. The genetics of autoimmune diseases: a networked perspective. Current opinion in immunology 21(6):596-605.

Baranzini SE, Wang J, Gibson RA, Galwey N, Naegelin Y, Barkhof F, Radue EW, Lindberg RL, Uitdehaag BM, Johnson MR and others. 2009. Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. Human molecular genetics 18(4):767-78.

Barbin G, Aigrot MS, Charles P, Foucher A, Grumet M, Schachner M, Zalc B, Lubetzki C. 2004. Axonal cell-adhesion molecule L1 in CNS myelination. Neuron glia biology 1(1):65-72.

Baron W, Colognato H, ffrench-Constant C. 2005. Integrin-growth factor interactions as regulators of oligodendroglial development and function. Glia 49(4):467-79.

Barres BA. 2008. The mystery and magic of glia: a perspective on their roles in health and disease. Neuron 60(3):430-40.

Barres BA, Lazar MA, Raff MC. 1994. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120(5):1097-108.

Barres BA, Raff MC. 1994. Control of oligodendrocyte number in the developing rat optic nerve. Neuron 12(5):935-42.

Bartoletti AL, Stevenson DK, Ostrander CR, Johnson JD. 1979. Pulmonary excretion of carbon monoxide in the human infant as an index of bilirubin production. I. Effects of gestational and postnatal age and some common neonatal abnormalities. The Journal of pediatrics 94(6):952-5.

Bashir K, Whitaker JN. 1999. Clinical and laboratory features of primary progressive and secondary progressive MS. Neurology 53(4):765-71.

Baumann N, Pham-Dinh D. 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiological reviews 81(2):871-927.

Bayer SA, Altman J. 1991. Development of the endopiriform nucleus and the claustrum in the rat brain. Neuroscience 45(2):391-412.

Begum R, Nur EKMS, Zaman MA. 2004. The role of Rho GTPases in the regulation of the rearrangement of actin cytoskeleton and cell movement. Experimental & molecular medicine 36(4):358-66.

Bellarosa C, Bortolussi G, Tiribelli C. 2009. The role of ABC transporters in protecting cells from bilirubin toxicity. Current pharmaceutical design 15(25):2884-92.

Bellefontaine N, Hanchate NK, Parkash J, Campagne C, de Seranno S, Clasadonte J, d'Anglemont de Tassigny X, Prevot V. 2011. Nitric oxide as key mediator of neuron-to-neuron and endothelia-to-glia communication involved in the neuroendocrine control of reproduction. Neuroendocrinology 93(2):74-89.

Benarroch EE. 2009. Oligodendrocytes: Susceptibility to injury and involvement in neurologic disease. Neurology 72(20):1779-85.

Benes FM. 2009. Neural circuitry models of schizophrenia: is it dopamine, GABA, glutamate, or something else? Biological psychiatry 65(12):1003-5.

Berger T, Walz W, Schnitzer J, Kettenmann H. 1992. GABA- and glutamate-activated currents in glial cells of the mouse corpus callosum slice. Journal of neuroscience research 31(1):21-7.

Bergles DE, Roberts JD, Somogyi P, Jahr CE. 2000. Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. Nature 405(6783):187-91.

Bergman I, Hirsch RP, Fria TJ, Shapiro SM, Holzman I, Painter MJ. 1985. Cause of hearing loss in the high-risk premature infant. The Journal of pediatrics 106(1):95-101.

Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nature cell biology 2(6):326-32.

Bessis A, Bechade C, Bernard D, Roumier A. 2007. Microglial control of neuronal death and synaptic properties. Glia 55(3):233-8.

Bhutani VK. 2001. Neonatal hyperbilirubinemia and the potential risk of subtle neurological dysfunction. Pediatric research 50(6):679-80.

Bhutani VK, Vilms RJ, Hamerman-Johnson L: Universal bilirubin screening for severe neonatal hyperbilirubinemia. 2010. Journal Perinatol 30(suppl):S6-15.

Bhutani VK, Johnson LH, Keren R. 2004. Diagnosis and management of hyperbilirubinemia in the term neonate: for a safer first week. Pediatric clinics of North America 51(4):843-61.

Bhutani VK, Stevenson DK. 2011. The need for technologies to prevent bilirubin-induced neurologic dysfunction syndrome. Seminars in perinatology 35(3):97-100.

Bielekova B, Martin R. 2004. Development of biomarkers in multiple sclerosis. Brain: a journal of neurology 127(Pt 7):1463-78.

Birling MC, Price J. 1998. A study of the potential of the embryonic rat telencephalon to generate oligodendrocytes. Developmental biology 193(1):100-13.

Bjelke B, Seiger A. 1989. Morphological distribution of MBP-like immunoreactivity in the brain during development. International journal of developmental neuroscience: the official journal of the International Society for Developmental Neuroscience 7(2):145-64.

Blackburn ST. 2003. Bilirubinmetabolism. In:Maternal, fetal, neonatal physiology: a clinical perspective. 2nd edition. St.Louis (MO): WBSaunders:652–76.

Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C, Wouters BG, Bell JC. 2004. Activating transcription factor 4 is translationally regulated by hypoxic stress. Molecular and cellular biology 24(17):7469-82.

Bogler O, Wren D, Barnett SC, Land H, Noble M. 1990. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proceedings of the National Academy of Sciences of the United States of America 87(16):6368-72.

Bourikas D, Mir A, Walmsley AR. 2010. LINGO-1-mediated inhibition of oligodendrocyte differentiation does not require the leucine-rich repeats and is reversed by p75(NTR) antagonists. Molecular and cellular neurosciences 45(4):363-9.

Boyer DB, Vidyasagar D. 1987. Serum indirect bilirubin levels and meconium passage in early fed normal newborns. Nursing research 36(3):174-8.

Bradl M, Lassmann H. 2010. Oligodendrocytes: biology and pathology. Acta neuropathologica 119(1):37-53.

Bretschneider T, Diez S, Anderson K, Heuser J, Clarke M, Muller-Taubenberger A, Kohler J, Gerisch G. 2004. Dynamic actin patterns and Arp2/3 assembly at the substrate-attached surface of motile cells. Current biology: CB 14(1):1-10.

Brites D. 2012. The envolving landscape of neurotoxicity by unconjugated bilirubin: role of glial cells and inflammation. Frontiers in Pharmacology 3(88):1-27.

Brites D, Fernandes A, Falcão AS, Gordo AC, Silva RFM, Brito MA. 2009. Biological risks for neurological abnormalities associated with hyperbilirubinemia. Journal of perinatology: official journal of the California Perinatal Association 29 Suppl 1:S8-13.

Brito MA, Brondino CD, Moura JJ, Brites D. 2001. Effects of bilirubin molecular species on membrane dynamic properties of human erythrocyte membranes: a spin label electron paramagnetic resonance spectroscopy study. Archives of biochemistry and biophysics 387(1):57-65.

Brito MA, Lima S, Fernandes A, Falcão AS, Silva RFM, Butterfield DA, Brites D. 2008. Bilirubin injury to neurons: contribution of oxidative stress and rescue by glycoursodeoxycholic acid. Neurotoxicology 29(2):259-69.

Brito MA, Vaz AR, Silva SL, Falcão AS, Fernandes A, Silva RFM, Brites D. 2010. N-methyl-aspartate receptor and neuronal nitric oxide synthase activation mediate bilirubin-induced neurotoxicity. Molecular medicine 16(9-10):372-80.

Brito MA, Zurolo E, Pereira P, Barroso C, Aronica E, Brites D. 2012. Cerebellar axon/myelin loss, angiogenic sprouting, and neuronal increase of vascular endothelial growth factor in a preterm infant with kernicterus. Journal of child neurology 27(5):615-24.

Brown AS. 2006. Prenatal infection as a risk factor for schizophrenia. Schizophrenia bulletin 32(2):200-2.

Bruses JL, Rutishauser U. 1998. Regulation of neural cell adhesion molecule polysialylation: evidence for nontranscriptional control and sensitivity to an intracellular pool of calcium. The Journal of cell biology 140(5):1177-86.

Buffo A, Vosko MR, Erturk D, Hamann GF, Jucker M, Rowitch D, Gotz M. 2005. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. Proceedings of the National Academy of Sciences of the United States of America 102(50):18183-8.

Bunge MB, Bunge RP, Pappas GD. 1962. Electron microscopic demonstration of connections between glia and myelin sheaths in the developing mammalian central nervous system. The Journal of cell biology 12:448-53.

Bunge RP. 1968. Glial cells and the central myelin sheath. Physiological reviews 48(1):197-251.

Bushong EA, Martone ME, Jones YZ, Ellisman MH. 2002. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. The Journal of neuroscience: the official journal of the Society for Neuroscience 22(1):183-92.

Butt AM. 2006. Neurotransmitter-mediated calcium signalling in oligodendrocyte physiology and pathology. Glia 54(7):666-75.

Byne W, Tatusov A, Yiannoulos G, Vong GS, Marcus S. 2008. Effects of mental illness and aging in two thalamic nuclei. Schizophrenia research 106(2-3):172-81.

Cai J, Qi Y, Hu X, Tan M, Liu Z, Zhang J, Li Q, Sander M, Qiu M. 2005. Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. Neuron 45(1):41-53.

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 415(6867):92-6.

Calza L, Fernandez M, Giuliani A, Aloe L, Giardino L. 2002. Thyroid hormone activates oligodendrocyte precursors and increases a myelin-forming protein and NGF content in the spinal cord during experimental allergic encephalomyelitis. Proceedings of the National Academy of Sciences of the United States of America 99(5):3258-63.

Cammer W. 2000. Effects of TNFalpha on immature and mature oligodendrocytes and their progenitors in vitro. Brain research 864(2):213-9.

Cao X, Deng X, May WS. 2003. Cleavage of Bax to p18 Bax accelerates stress-induced apoptosis, and a cathepsin-like protease may rapidly degrade p18 Bax. Blood 102(7):2605-14.

Chang FY, Lee CC, Huang CC, Hsu KS. 2009. Unconjugated bilirubin exposure impairs hippocampal long-term synaptic plasticity. PloS one 4(6):e5876.

Charles P, Hernandez MP, Stankoff B, Aigrot MS, Colin C, Rougon G, Zalc B, Lubetzki C. 2000. Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule. Proceedings of the National Academy of Sciences of the United States of America 97(13):7585-90.

Cheepsunthorn P, Palmer C, Connor JR. 1998. Cellular distribution of ferritin subunits in postnatal rat brain. The Journal of comparative neurology 400(1):73-86.

Chen HC, Wang CH, Tsan KW, Chen YC. 1971. An electron microscopic and radioautographic study on experimental kernicterus. II. Bilirubin movement within neurons and release of waste products via astroglia. The American journal of pathology 64(1):45-66.

Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA. 2001. Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. The Journal of biological chemistry 276(33):30724-8.

Chen X, Shen J, Prywes R. 2002. The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. The Journal of biological chemistry 277(15):13045-52.

Chen Y, Ghosh A. 2005. Regulation of dendritic development by neuronal activity. Journal of neurobiology 64(1):4-10.

Chew LJ, King WC, Kennedy A, Gallo V. 2005. Interferon-gamma inhibits cell cycle exit in differentiating oligodendrocyte progenitor cells. Glia 52(2):127-43.

Chittajallu R, Aguirre A, Gallo V. 2004. NG2-positive cells in the mouse white and grey matter display distinct physiological properties. The Journal of physiology 561(Pt 1):109-22.

Claireaux AE. 2003. Pathology of human kernicterus. In A. Sass-Kortsak (Ed.), Kernicterus: A Report Based on a Symposium Held at the IX International Congress of Paediatrics (pp. 1961:1140). Toronto: University of Toronto Press.

Coffey JC, McDermott KW. 1997. The regional distribution of myelin oligodendrocyte glycoprotein (MOG) in the developing rat CNS: an in vivo immunohistochemical study. Journal of neurocytology 26(3):149-61.

Cohen RI, Chandross KJ. 2000. Fibroblast growth factor-9 modulates the expression of myelin related proteins and multiple fibroblast growth factor receptors in developing oligodendrocytes. Journal of neuroscience research 61(3):273-87.

Coman I, Barbin G, Charles P, Zalc B, Lubetzki C. 2005. Axonal signals in central nervous system myelination, demyelination and remyelination. Journal of the neurological sciences 233(1-2):67-71.

Compston A. 2006. Making progress on the natural history of multiple sclerosis. Brain: a journal of neurology 129(Pt 3):561-3.

Compston A, Coles A. 2002. Multiple sclerosis. Lancet 359(9313):1221-31.

Compston A, Coles A. 2008. Multiple sclerosis. Lancet 372(9648):1502-17.

Conlee JW, Shapiro SM. 1997. Development of cerebellar hypoplasia in jaundiced Gunn rats: a quantitative light microscopic analysis. Acta neuropathologica 93(5):450-60.

Connor JR, Menzies SL. 1996. Relationship of iron to oligodendrocytes and myelination. Glia 17(2):83-93.

Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E. 2006. Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. Stem cells 24(4):1001-10.

Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y. 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438(7070):1017-21.

Craig A, Ling Luo N, Beardsley DJ, Wingate-Pearse N, Walker DW, Hohimer AR, Back SA. 2003. Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. Experimental neurology 181(2):231-40.

Dalman C, Cullberg J. 1999. Neonatal hyperbilirubinaemia - a vulnerability factor for mental disorder? Acta psychiatrica Scandinavica 100(6):469-71.

Dalmau I, Finsen B, Zimmer J, Gonzalez B, Castellano B. 1998. Development of microglia in the postnatal rat hippocampus. Hippocampus 8(5):458-74.

Daood MJ, McDonagh AF, Watchko JF. 2009. Calculated free bilirubin levels and neurotoxicity. Journal of perinatology : official journal of the California Perinatal Association 29 Suppl 1:S14-9.

Daskalakis ZJ, Christensen BK, Fitzgerald PB, Chen R. 2008. Dysfunctional neural plasticity in patients with schizophrenia. Archives of general psychiatry 65(4):378-85.

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. 2005. ATP mediates rapid microglial response to local brain injury in vivo. Nature neuroscience 8(6):752-8.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. Cell 103(2):239-52.

De Biase LM, Nishiyama A, Bergles DE. 2010. Excitability and synaptic communication within the oligodendrocyte lineage. The Journal of neuroscience: the official journal of the Society for Neuroscience 30(10):3600-11.

Dean JM, Moravec MD, Grafe M, Abend N, Ren J, Gong X, Volpe JJ, Jensen FE, Hohimer AR, Back SA. 2011. Strain-specific differences in perinatal rodent oligodendrocyte lineage progression and its correlation with human. Developmental neuroscience 33(3-4):251-60.

Deng W, Rosenberg PA, Volpe JJ, Jensen FE. 2003. Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. Proceedings of the National Academy of Sciences of the United States of America 100(11):6801-6.

Deniaud A, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C, Brenner C. 2008. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. Oncogene 27(3):285-99.

Dennery PA, Seidman DS, Stevenson DK: Neonatal hyperbilirubinemia. 2001. The New England Journal of Medicine 344:581-590.

Derivery E, Gautreau A. 2010. Generation of branched actin networks: assembly and regulation of the N-WASP and WAVE molecular machines. BioEssays: news and reviews in molecular, cellular and developmental biology 32(2):119-31.

Doherty P, Walsh FS. 1996. CAM-FGF Receptor Interactions: A Model for Axonal Growth. Molecular and cellular neurosciences 8(2/3):99-111.

Domercq M, Sánchez-Gómez MV, Sherwin C, Etxebarria E, Fern R, Matute C. 2007. System xc- and glutamate transporter inhibition mediates microglial toxicity to oligodendrocytes. Journal of immunology 178(10):6549-56.

Dorph-Petersen KA, Delevich KM, Marcsisin MJ, Zhang W, Sampson AR, Gundersen HJ, Lewis DA, Sweet RA. 2009. Pyramidal neuron number in layer 3 of primary auditory cortex of subjects with schizophrenia. Brain research 1285:42-57.

Dorph-Petersen KA, Pierri JN, Wu Q, Sampson AR, Lewis DA. 2007. Primary visual cortex volume and total neuron number are reduced in schizophrenia. The Journal of comparative neurology 501(2):290-301.

Dussault JH, Ruel J. 1987. Thyroid hormones and brain development. Annual review of physiology 49:321-34.

Ellison-Wright I, Bullmore E. 2010. Anatomy of bipolar disorder and schizophrenia: a meta-analysis. Schizophrenia research 117(1):1-12.

Erschbamer MK, Hofstetter CP, Olson L. 2005. RhoA, RhoB, RhoC, Rac1, Cdc42, and Tc10 mRNA levels in spinal cord, sensory ganglia, and corticospinal tract neurons and long-lasting specific changes following spinal cord injury. The Journal of comparative neurology 484(2):224-33.

Escartin C, Bonvento G. 2008. Targeted activation of astrocytes: a potential neuroprotective strategy. Molecular neurobiology 38(3):231-41.

Falcão AS, Bellarosa C, Fernandes A, Brito MA, Silva RFM, Tiribelli C, Brites D. 2007a. Role of multidrug resistance-associated protein 1 expression in the in vitro susceptibility of rat nerve cell to unconjugated bilirubin. Neuroscience 144(3):878-88.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2005. Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. Neurobiology of disease 20(2):199-206.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta neuropathologica 112(1):95-105.

Falcão AS, Silva RFM, Fernandes A, Brito MA, Brites D. 2007b. Influence of hypoxia and ischemia preconditioning on bilirubin damage to astrocytes. Brain research 1149:191-9.

Farahani R, Pina-Benabou MH, Kyrozis A, Siddiq A, Barradas PC, Chiu FC, Cavalcante LA, Lai JC, Stanton PK, Rozental R. 2005. Alterations in metabolism and gap junction expression may determine the role of astrocytes as "good samaritans" or executioners. Glia 50(4):351-61.

Farber K, Kettenmann H. 2006. Purinergic signaling and microglia. Pflugers Archiv: European journal of physiology 452(5):615-21.

Fatemi SH, Folsom TD. 2009. The neurodevelopmental hypothesis of schizophrenia, revisited. Schizophrenia bulletin 35(3):528-48.

Featherstone RE, Kapur S, Fletcher PJ. 2007. The amphetamine-induced sensitized state as a model of schizophrenia. Progress in neuro-psychopharmacology & biological psychiatry 31(8):1556-71.

Fellin T, Halassa MM, Terunuma M, Succol F, Takano H, Frank M, Moss SJ, Haydon PG. 2009. Endogenous nonneuronal modulators of synaptic transmission control cortical slow oscillations in vivo. Proceedings of the National Academy of Sciences of the United States of America 106(35):15037-42.

Ferguson B, Matyszak MK, Esiri MM, Perry VH. 1997. Axonal damage in acute multiple sclerosis lesions. Brain : a journal of neurology 120 (Pt 3):393-9.

Fern R, Moller T. 2000. Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. The Journal of neuroscience: the official journal of the Society for Neuroscience 20(1):34-42.

Fernandes A, Barateiro A, Falcão AS, Silva SL, Vaz AR, Brito MA, Silva RFM, Brites D. 2011. Astrocyte reactivity to unconjugated bilirubin requires TNF-alpha and IL-1beta receptor signaling pathways. Glia 59(1):14-25.

Fernandes A, Falcão AS, Abranches E, Bekman E, Henrique D, Lanier LM, Brites D. 2009. Bilirubin as a determinant for altered neurogenesis, neuritogenesis, and synaptogenesis. Developmental neurobiology 69(9):568-82.

Fernandes A, Falcão AS, Silva RFM, Brito MA, Brites D. 2007. MAPKs are key players in mediating cytokine release and cell death induced by unconjugated bilirubin in cultured rat cortical astrocytes. The European journal of neuroscience 25(4):1058-68.

Fernandes A, Falcão AS, Silva RFM, Gordo AC, Gama MJ, Brito MA, Brites D. 2006. Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. Journal of neurochemistry 96(6):1667-79.

Fernandes A, Silva RFM, Falcão AS, Brito MA, Brites D. 2004. Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. Journal of neuroimmunology 153(1-2):64-75.

Fex Svenningsen A, Shan WS, Colman DR, Pedraza L. 2003. Rapid method for culturing embryonic neuron-glial cell cocultures. Journal of neuroscience research 72(5):565-73.

Fields RD. 2008. White matter in learning, cognition and psychiatric disorders. Trends in neurosciences 31(7):361-70.

Fields RD, Stevens-Graham B. 2002. New insights into neuron-glia communication. Science 298(5593):556-62.

Fiske BK, Brunjes PC. 2000. Microglial activation in the developing rat olfactory bulb. Neuroscience 96(4):807-15.

Fogarty M, Richardson WD, Kessaris N. 2005. A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. Development 132(8):1951-9.

Foussias G, Remington G. 2010. Negative symptoms in schizophrenia: avolition and Occam's razor. Schizophrenia bulletin 36(2):359-69.

Franciotta D, Salvetti M, Lolli F, Serafini B, Aloisi F. 2008. B cells and multiple sclerosis. Lancet neurology 7(9):852-8.

Fressinaud C, Vallat JM, Labourdette G. 1995. Basic fibroblast growth factor down-regulates myelin basic protein gene expression and alters myelin compaction of mature oligodendrocytes in vitro. Journal of neuroscience research 40(3):285-93.

Froger N, Orellana JA, Calvo CF, Amigou E, Kozoriz MG, Naus CC, Saez JC, Giaume C. 2010. Inhibition of cytokine-induced connexin43 hemichannel activity in astrocytes is neuroprotective. Molecular and cellular neurosciences 45(1):37-46.

Gallo V, Mangin JM, Kukley M, Dietrich D. 2008. Synapses on NG2-expressing progenitors in the brain: multiple functions? The Journal of physiology 586(16):3767-81.

Gard AL, Pfeiffer SE. 1989. Oligodendrocyte progenitors isolated directly from developing telencephalon at a specific phenotypic stage: myelinogenic potential in a defined environment. Development 106(1):119-32.

Gardener H, Spiegelman D, Buka SL. 2011. Perinatal and neonatal risk factors for autism: a comprehensive meta-analysis. Pediatrics 128(2):344-55.

Gartner LM. 1994. On the question of the relationship between breastfeeding and jaundice in the first 5 days of life. Seminars in perinatology 18(6):502-9.

Gazzin S, Berengeno AL, Strazielle N, Fazzari F, Raseni A, Ostrow JD, Wennberg R, Ghersi-Egea JF, Tiribelli C. 2011. Modulation of Mrp1 (ABCc1) and Pgp (ABCb1) by bilirubin at the blood-CSF and blood-brain barriers in the Gunn rat. PloS one 6(1):e16165.

Genc S, Genc K, Kumral A, Baskin H, Ozkan H. 2003. Bilirubin is cytotoxic to rat oligodendrocytes in vitro. Brain research 985(2):135-41.

Gkoltsiou K, Tzoufi M, Counsell S, Rutherford M, Cowan F. 2008. Serial brain MRI and ultrasound findings: relation to gestational age, bilirubin level, neonatal neurologic status and neurodevelopmental outcome in infants at risk of kernicterus. Early human development 84(12):829-38.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. 2010. Mechanisms underlying inflammation in neurodegeneration. Cell 140(6):918-34.

Goddard DR, Berry M, Kirvell SL, Butt AM. 2001. Fibroblast growth factor-2 inhibits myelin production by oligodendrocytes in vivo. Molecular and cellular neurosciences 18(5):557-69.

Gokhan S, Marin-Husstege M, Yung SY, Fontanez D, Casaccia-Bonnefil P, Mehler MF. 2005. Combinatorial profiles of oligodendrocyte-selective classes of transcriptional regulators differentially modulate myelin basic protein gene expression. The Journal of neuroscience : the official journal of the Society for Neuroscience 25(36):8311-21.

Gordo AC, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Unconjugated bilirubin activates and damages microglia. Journal of neuroscience research 84(1):194-201.

Gordon GR, Mulligan SJ, MacVicar BA. 2007. Astrocyte control of the cerebrovasculature. Glia 55(12):1214-21.

Gorlach A, Klappa P, Kietzmann T. 2006. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxidants & redox signaling 8(9-10):1391-418.

Gourley GR. 1997. Bilirubin metabolism and kernicterus. Advances in pediatrics 44:173-229.

Gourley GR. 2002. Breast-feeding, neonatal jaundice and kernicterus. Seminars in neonatology: SN 7(2):135-41.

Gravel M, Robert F, Kottis V, Gallouzi IE, Pelletier J, Braun PE. 2009. 2',3'-Cyclic nucleotide 3'-phosphodiesterase: a novel RNA-binding protein that inhibits protein synthesis. Journal of neuroscience research 87(5):1069-79.

Greer JM, Csurhes PA, Cameron KD, McCombe PA, Good MF, Pender MP. 1997. Increased immunoreactivity to two overlapping peptides of myelin proteolipid protein in multiple sclerosis. Brain : a journal of neurology 120 (Pt 8):1447-60.

Grima G, Benz B, Parpura V, Cuenod M, Do KQ. 2003. Dopamine-induced oxidative stress in neurons with glutathione deficit: implication for schizophrenia. Schizophrenia research 62(3):213-24.

Grinspan JB, Stern JL, Franceschini B, Pleasure D. 1993. Trophic effects of basic fibroblast growth factor (bFGF) on differentiated oligodendroglia: a mechanism for regeneration of the oligodendroglial lineage. Journal of neuroscience research 36(6):672-80.

Grojean S, Koziel V, Vert P, Daval JL. 2000. Bilirubin induces apoptosis via activation of NMDA receptors in developing rat brain neurons. Experimental neurology 166(2):334-41.

Grojean S, Lievre V, Koziel V, Vert P, Daval JL. 2001. Bilirubin exerts additional toxic effects in hypoxic cultured neurons from the developing rat brain by the recruitment of glutamate neurotoxicity. Pediatric research 49(4):507-13.

Gurba PE, Zand R. 1974. Bilirubin binding to myelin basic protein, histones and its inhibition in vitro of cerebellar protein synthesis. Biochemical and biophysical research communications 58(4):1142-7.

Hadley SD, Goshgarian HG. 1997. Altered immunoreactivity for glial fibrillary acidic protein in astrocytes within 1 h after cervical spinal cord injury. Experimental neurology 146(2):380-7.

Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, Haroutunian V, Fienberg AA. 2001. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proceedings of the National Academy of Sciences of the United States of America 98(8):4746-51.

Hall A, Giese NA, Richardson WD. 1996. Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors. Development 122(12):4085-94.

Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature neuroscience 10(11):1387-94.

Hansen T, Tommarello S, Allen J. 2001. Subcellular localization of bilirubin in rat brain after in vivo i.v. administration of [3H]bilirubin. Pediatric research 49(2):203-7.

Hansen TW. 2000. Pioneers in the scientific study of neonatal jaundice and kernicterus. Pediatrics 106(2):E15.

Hansen TW, Maynard EC, Cashore WJ, Oh W. 1993. Endotoxemia and brain bilirubin in the rat. Biology of the neonate 63(3):171-6.

Hardy RJ, Friedrich VL, Jr. 1996. Progressive remodeling of the oligodendrocyte process arbor during myelinogenesis. Developmental neuroscience 18(4):243-54.

Harrison PJ, Weinberger DR. 2005. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Molecular psychiatry 10(1):40-68.

Hartman BK, Agrawal HC, Kalmbach S, Shearer WT. 1979. A comparative study of the immunohistochemical localization of basic protein to myelin and oligodendrocytes in rat and chicken brain. The Journal of comparative neurology 188(2):273-90.

Hatten ME. 1999. Central nervous system neuronal migration. Annual review of neuroscience 22:511-39.

Hatzenbuehler L, Zaidi AK, Sundar S, Sultana S, Abbasi F, Rizvi A, Darmstadt GL. 2010. Validity of neonatal jaundice evaluation by primary health-care workers and physicians in Karachi, Pakistan. Journal of perinatology: official journal of the California Perinatal Association 30(9):616-21.

Haynes RL, Baud O, Li J, Kinney HC, Volpe JJ, Folkerth DR. 2005. Oxidative and nitrative injury in periventricular leukomalacia: a review. Brain pathology 15(3):225-33.

Heimer L. 2000. Basal forebrain in the context of schizophrenia. Brain research Brain research reviews 31(2-3):205-35.

Henry LP, Amminger GP, Harris MG, Yuen HP, Harrigan SM, Prosser AL, Schwartz OS, Farrelly SE, Herrman H, Jackson HJ and others. 2010. The EPPIC follow-up study of first-episode psychosis: longer-term clinical and functional outcome 7 years after index admission. The Journal of clinical psychiatry 71(6):716-28.

Hof PR, Haroutunian V, Friedrich VL, Jr., Byne W, Buitron C, Perl DP, Davis KL. 2003. Loss and altered spatial distribution of oligodendrocytes in the superior frontal gyrus in schizophrenia. Biological psychiatry 53(12):1075-85.

Hoffman KL, Duncan ID. 1995. Canine oligodendrocytes undergo morphological changes in response to basic fibroblast growth factor (bFGF) in vitro. Glia 14(1):33-42.

Hohlfeld R. 2010. 'Gimme five': future challenges in multiple sclerosis. ECTRIMS Lecture 2009. Multiple sclerosis 16(1):3-14.

Hong M, Li M, Mao C, Lee AS. 2004. Endoplasmic reticulum stress triggers an acute proteasome-dependent degradation of ATF6. Journal of cellular biochemistry 92(4):723-32.

Horiuchi M, Itoh A, Pleasure D, Itoh T. 2006. MEK-ERK signaling is involved in interferon-gamma-induced death of oligodendroglial progenitor cells. The Journal of biological chemistry 281(29):20095-106.

Hortsch M. 1996. The L1 family of neural cell adhesion molecules: old proteins performing new tricks. Neuron 17(4):587-93.

Hortsch M. 2000. Structural and functional evolution of the L1 family: are four adhesion molecules better than one? Molecular and cellular neurosciences 15(1):1-10.

Hovelmeyer N, Hao Z, Kranidioti K, Kassiotis G, Buch T, Frommer F, von Hoch L, Kramer D, Minichiello L, Kollias G and others. 2005. Apoptosis of oligodendrocytes via Fas and TNF-R1 is a key event in the induction of experimental autoimmune encephalomyelitis. Journal of immunology 175(9):5875-84.

Hua JY, Smith SJ. 2004. Neural activity and the dynamics of central nervous system development. Nature neuroscience 7(4):327-32.

Huang YH, Bergles DE. 2004. Glutamate transporters bring competition to the synapse. Current opinion in neurobiology 14(3):346-52.

Huberman AD, Feller MB, Chapman B. 2008. Mechanisms underlying development of visual maps and receptive fields. Annual review of neuroscience 31:479-509.

Huppi PS, Warfield S, Kikinis R, Barnes PD, Zientara GP, Jolesz FA, Tsuji MK, Volpe JJ. 1998. Quantitative magnetic resonance imaging of brain development in premature and mature newborns. Annals of neurology 43(2):224-35.

Hyman CB, Keaster J, Hanson V, Harris I, Sedgwick R, Wursten H, Wright AR. 1969. CNS abnormalities after neonatal hemolytic disease or hyperbilirubinemia. A prospective study of 405 patients. American journal of diseases of children 117(4):395-405.

Iadecola C, Nedergaard M. 2007. Glial regulation of the cerebral microvasculature. Nature neuroscience 10(11):1369-76.

Ibarrola N, Mayer-Proschel M, Rodriguez-Pena A, Noble M. 1996. Evidence for the existence of at least two timing mechanisms that contribute to oligodendrocyte generation in vitro. Developmental biology 180(1):1-21.

Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S. 1996. A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. Biochemical and biophysical research communications 224(3):855-62.

Imai Y, Kohsaka S. 2002. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. Glia 40(2):164-74.

Ishibashi T, Dakin KA, Stevens B, Lee PR, Kozlov SV, Stewart CL, Fields RD. 2006. Astrocytes promote myelination in response to electrical impulses. Neuron 49(6):823-32.

Itoh T, Beesley J, Itoh A, Cohen AS, Kavanaugh B, Coulter DA, Grinspan JB, Pleasure D. 2002. AMPA glutamate receptor-mediated calcium signaling is transiently enhanced during development of oligodendrocytes. Journal of neurochemistry 81(2):390-402.

Jakovcevski I, Filipovic R, Mo Z, Rakic S, Zecevic N. 2009. Oligodendrocyte development and the onset of myelination in the human fetal brain. Frontiers in neuroanatomy 3:5.

Jakovcevski I, Mo Z, Zecevic N. 2007. Down-regulation of the axonal polysialic acid-neural cell adhesion molecule expression coincides with the onset of myelination in the human fetal forebrain. Neuroscience 149(2):328-37.

Jenkins HG, Ikeda H. 1992. Tumour necrosis factor causes an increase in axonal transport of protein and demyelination in the mouse optic nerve. Journal of the neurological sciences 108(1):99-104.

Jessell TM. 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nature reviews Genetics 1(1):20-9.

Jessen KR. 2004. Glial cells. The international journal of biochemistry & cell biology 36(10):1861-7.

Jew JY, Williams TH. 1977. Ultrastructural aspects of bilirubin encephalopathy in cochlear nuclei of the Gunn rat. Journal of anatomy 124(Pt 3):599-614.

Jiang F, Frederick TJ, Wood TL. 2001. IGF-I synergizes with FGF-2 to stimulate oligodendrocyte progenitor entry into the cell cycle. Developmental biology 232(2):414-23.

Johnson L, Bhutani VK. 2011. The clinical syndrome of bilirubin-induced neurologic dysfunction. Seminars in perinatology 35(3):101-13.

Jurewicz A, Matysiak M, Tybor K, Kilianek L, Raine CS, Selmaj K. 2005. Tumour necrosis factor-induced death of adult human oligodendrocytes is mediated by apoptosis inducing factor. Brain : a journal of neurology 128(Pt 11):2675-88.

Kadi L, Selvaraju R, de Lys P, Proudfoot AE, Wells TN, Boschert U. 2006. Differential effects of chemokines on oligodendrocyte precursor proliferation and myelin formation in vitro. Journal of neuroimmunology 174(1-2):133-46.

Kanaan RA, Kim JS, Kaufmann WE, Pearlson GD, Barker GJ, McGuire PK. 2005. Diffusion tensor imaging in schizophrenia. Biological psychiatry 58(12):921-9.

Kaplan M, Hammerman C. 2005. Understanding severe hyperbilirubinemia and preventing kernicterus: adjuncts in the interpretation of neonatal serum bilirubin. Clinica chimica acta; international journal of clinical chemistry 356(1-2):9-21.

Káradóttir R, Attwell D. 2007. Neurotransmitter receptors in the life and death of oligodendrocytes. Neuroscience 145(4):1426-38.

Káradóttir R, Cavelier P, Bergersen LH, Attwell D. 2005. NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. Nature 438(7071):1162-6.

Katz LC, Shatz CJ. 1996. Synaptic activity and the construction of cortical circuits. Science 274(5290):1133-8.

Kaufman RJ. 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes & development 13(10):1211-33.

Kaur C, Hao AJ, Wu CH, Ling EA. 2001. Origin of microglia. Microscopy research and technique 54(1):2-9.

Kawade N, Onishi S. 1981. The prenatal and postnatal development of UDP-glucuronyltransferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. The Biochemical journal 196(1):257-60.

Keino H, Kashiwamata S. 1989. Critical period of bilirubin-induced cerebellar hypoplasia in a new Sprague-Dawley strain of jaundiced Gunn rats. Neuroscience research 6(3):209-15.

Kempiak SJ, Yamaguchi H, Sarmiento C, Sidani M, Ghosh M, Eddy RJ, Desmarais V, Way M, Condeelis J, Segall JE. 2005. A neural Wiskott-Aldrich Syndrome protein-mediated pathway for localized activation of actin polymerization that is regulated by cortactin. The Journal of biological chemistry 280(7):5836-42.

Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD. 2006. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nature neuroscience 9(2):173-9.

Kessaris N, Pringle N, Richardson WD. 2001. Ventral neurogenesis and the neuron-glial switch. Neuron 31(5):677-80.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. 2011. Physiology of microglia. Physiological reviews 91(2):461-553.

Kettenmann HR, BR. 2005. Neuroglia: USA: Oxford University Press.

Kim HJ, DiBernardo AB, Sloane JA, Rasband MN, Solomon D, Kosaras B, Kwak SP, Vartanian TK. 2006. WAVE1 is required for oligodendrocyte morphogenesis and normal CNS

myelination. The Journal of neuroscience: the official journal of the Society for Neuroscience 26(21):5849-59.

Kim J, Jung Y. 2011. Different expressions of AQP1, AQP4, eNOS, and VEGF proteins in ischemic versus non-ischemic cerebropathy in rats: potential roles of AQP1 and eNOS in hydrocephalic and vasogenic edema formation. Anatomy & cell biology 44(4):295-303.

Kim S, Steelman AJ, Koito H, Li J. 2011. Astrocytes promote TNF-mediated toxicity to oligodendrocyte precursors. Journal of neurochemistry 116(1):53-66.

Kim SU, de Vellis J. 2005. Microglia in health and disease. Journal of neuroscience research 81(3):302-13.

Kiss JZ, Rougon G. 1997. Cell biology of polysialic acid. Current opinion in neurobiology 7(5):640-6.

Kiss JZ, Wang C, Olive S, Rougon G, Lang J, Baetens D, Harry D, Pralong WF. 1994. Activity-dependent mobilization of the adhesion molecule polysialic NCAM to the cell surface of neurons and endocrine cells. The EMBO journal 13(22):5284-92.

Kornyei Z, Szlavik V, Szabo B, Gocza E, Czirok A, Madarasz E. 2005. Humoral and contact interactions in astroglia/stem cell co-cultures in the course of glia-induced neurogenesis. Glia 49(3):430-44.

Kubicki M, McCarley R, Westin CF, Park HJ, Maier S, Kikinis R, Jolesz FA, Shenton ME. 2007. A review of diffusion tensor imaging studies in schizophrenia. Journal of psychiatric research 41(1-2):15-30.

Kubicki M, Park H, Westin CF, Nestor PG, Mulkern RV, Maier SE, Niznikiewicz M, Connor EE, Levitt JJ, Frumin M and others. 2005. DTI and MTR abnormalities in schizophrenia: analysis of white matter integrity. NeuroImage 26(4):1109-18.

Kuhlmann T, Lingfeld G, Bitsch A, Schuchardt J, Bruck W. 2002. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain : a journal of neurology 125(Pt 10):2202-12.

Kukley M, Nishiyama A, Dietrich D. 2010. The fate of synaptic input to NG2 glial cells: neurons specifically downregulate transmitter release onto differentiating oligodendroglial cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 30(24):8320-31.

Kumar S, Kahn MA, Dinh L, de Vellis J. 1998. NT-3-mediated TrkC receptor activation promotes proliferation and cell survival of rodent progenitor oligodendrocyte cells in vitro and in vivo. Journal of neuroscience research 54(6):754-65.

Landmesser L, Dahm L, Tang JC, Rutishauser U. 1990. Polysialic acid as a regulator of intramuscular nerve branching during embryonic development. Neuron 4(5):655-67.

Lappe-Siefke C, Goebbels S, Gravel M, Nicksch E, Lee J, Braun PE, Griffiths IR, Nave KA. 2003. Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. Nature genetics 33(3):366-74.

Lawson LJ, Perry VH, Dri P, Gordon S. 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39(1):151-70.

Lee AH, Iwakoshi NN, Glimcher LH. 2003. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Molecular and cellular biology 23(21):7448-59.

Lee AH, Scapa EF, Cohen DE, Glimcher LH. 2008. Regulation of hepatic lipogenesis by the transcription factor XBP1. Science 320(5882):1492-6.

Lee AS. 2005. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35(4):373-81.

Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K, Kaufman RJ. 2002. IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes & development 16(4):452-66.

Lewis DA, Sweet RA. 2009. Schizophrenia from a neural circuitry perspective: advancing toward rational pharmacological therapies. The Journal of clinical investigation 119(4):706-16.

Li J, Baud O, Vartanian T, Volpe JJ, Rosenberg PA. 2005. Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. Proceedings of the National Academy of Sciences of the United States of America 102(28):9936-41.

Li J, Ramenaden ER, Peng J, Koito H, Volpe JJ, Rosenberg PA. 2008. Tumor necrosis factor alpha mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present. The Journal of neuroscience : the official journal of the Society for Neuroscience 28(20):5321-30.

Li S, Stys PK. 2000. Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter. The Journal of neuroscience : the official journal of the Society for Neuroscience 20(3):1190-8.

Li W, Zhang B, Tang J, Cao Q, Wu Y, Wu C, Guo J, Ling EA, Liang F. 2007. Sirtuin 2, a mammalian homolog of yeast silent information regulator-2 longevity regulator, is an oligodendroglial protein that decelerates cell differentiation through deacetylating alpha-tubulin. The Journal of neuroscience: the official journal of the Society for Neuroscience 27(10):2606-16.

Liang X, Draghi NA, Resh MD. 2004. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience 24(32):7140-9.

Liaury K, Miyaoka T, Tsumori T, Furuya M, Wake R, Ieda M, Tsuchie K, Taki M, Ishihara K, Tanra AJ and others. 2012. Morphological features of microglial cells in the hippocampal dentate gyrus of Gunn rat: a possible schizophrenia animal model. Journal of neuroinflammation 9:56.

Liguori M, Marrosu MG, Pugliatti M, Giuliani F, De Robertis F, Cocco E, Zimatore GB, Livrea P, Trojano M. 2000. Age at onset in multiple sclerosis. Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology 21(4 Suppl 2):S825-9.

Lim DA, Alvarez-Buylla A. 1999. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proceedings of the National Academy of Sciences of the United States of America 96(13):7526-31.

Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergaard M. 1998. Gap-junction-mediated propagation and amplification of cell injury. Nature neuroscience 1(6):494-500.

Lin SC, Bergles DE. 2004. Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus. Nature neuroscience 7(1):24-32.

Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S, Grace AA. 2008. Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. Trends in neurosciences 31(5):234-42.

Liu W, Tang Y, Feng J. 2011. Cross talk between activation of microglia and astrocytes in pathological conditions in the central nervous system. Life sciences 89(5-6):141-6.

Liu W, Wang CH, Cui Y, Mo LQ, Zhi JL, Sun SN, Wang YL, Yu HM, Zhao CM, Feng JQ and others. 2006. Inhibition of neuronal nitric oxide synthase antagonizes morphine antinociceptive tolerance by decreasing activation of p38 MAPK in the spinal microglia. Neuroscience letters 410(3):174-7.

Liu Z, Hu X, Cai J, Liu B, Peng X, Wegner M, Qiu M. 2007. Induction of oligodendrocyte differentiation by Olig2 and Sox10: evidence for reciprocal interactions and dosage-dependent mechanisms. Developmental biology 302(2):683-93.

Lossi L, Alasia S, Salio C, Merighi A. 2009. Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. Progress in neurobiology 88(4):221-45.

Lu PD, Harding HP, Ron D. 2004. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. The Journal of cell biology 167(1):27-33.

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 109(1):75-86.

Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH. 2000. Sonic hedgehog-regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron 25(2):317-29.

Ludwin SK. 1997. The pathobiology of the oligodendrocyte. Journal of neuropathology and experimental neurology 56(2):111-24.

Lunn KF, Baas PW, Duncan ID. 1997. Microtubule organization and stability in the oligodendrocyte. The Journal of neuroscience: the official journal of the Society for Neuroscience 17(13):4921-32.

Lynch MA. 2009. The multifaceted profile of activated microglia. Molecular neurobiology 40(2):139-56.

Maden A, MacMahon JR, Stevenson DK. 2005. Neonatal hyperbilirubinemia. In: Taeusch HW, Ballard RA, Gleason CA, editors. Avery's diseases of the newborn. 8th edition. Philadelphia: Elsevier Saunders:1226-56.

Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, Ohashi K, Mizuno K, Narumiya S. 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science 285(5429):895-8.

Mahad D, Lassmann H, Turnbull D. 2008. Review: Mitochondria and disease progression in multiple sclerosis. Neuropathology and applied neurobiology 34(6):577-89.

Mahad DJ, Ziabreva I, Campbell G, Lax N, White K, Hanson PS, Lassmann H, Turnbull DM. 2009. Mitochondrial changes within axons in multiple sclerosis. Brain: a journal of neurology 132(Pt 5):1161-74.

Maimburg RD, Bech BH, Vaeth M, Moller-Madsen B, Olsen J. 2010. Neonatal jaundice, autism, and other disorders of psychological development. Pediatrics 126(5):872-8.

Maisels MJ. 2005. Jaundice. In: MacDonald MG, Mullett MD, Seshia MMK, editors. Avery's neonatology: pathophysiology & management of the newborn. 6th edition. Philadelphia: Lippincott Williams & Wilkins; 2005. p. 768–846.

Maisels MJ, Kring E. 2006. Transcutaneous bilirubin levels in the first 96 hours in a normal newborn population of > or = 35 weeks' gestation. Pediatrics 117(4):1169-73.

Maisels MJ, McDonagh AF. 2008. Phototherapy for neonatal jaundice. The New England journal of medicine 358(9):920-8.

Maisels MJ, Pathak A, Nelson NM, Nathan DG, Smith CA. 1971. Endogenous production of carbon monoxide in normal and erythroblastotic newborn infants. The Journal of clinical investigation 50(1):1-8.

Maisels MJ, Watchko JF. 2003. Treatment of jaundice in low birthweight infants. Archives of disease in childhood Fetal and neonatal edition 88(6):F459-63.

Malhotra JD, Kaufman RJ. 2007. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxidants & redox signaling 9(12):2277-93.

Marquardt T, Pfaff SL. 2001. Cracking the transcriptional code for cell specification in the neural tube. Cell 106(6):651-4.

Martich-Kriss V, Kollias SS, Ball WS, Jr. 1995. MR findings in kernicterus. AJNR American journal of neuroradiology 16(4 Suppl):819-21.

Martinez R, Gomes FC. 2002. Neuritogenesis induced by thyroid hormone-treated astrocytes is mediated by epidermal growth factor/mitogen-activated protein kinase-phosphatidylinositol 3-kinase pathways and involves modulation of extracellular matrix proteins. The Journal of biological chemistry 277(51):49311-8.

Martinez R, Gomes FC. 2005. Proliferation of cerebellar neurons induced by astrocytes treated with thyroid hormone is mediated by a cooperation between cell contact and soluble

factors and involves the epidermal growth factor-protein kinase a pathway. Journal of neuroscience research 80(3):341-9.

Matute C. 2006. Oligodendrocyte NMDA receptors: a novel therapeutic target. Trends in molecular medicine 12(7):289-92.

Matute C. 2010. Calcium dyshomeostasis in white matter pathology. Cell calcium 47(2):150-7.

Matute C, Alberdi E, Domercq M, Sánchez-Gómez MV, Perez-Samartin A, Rodriguez-Antiguedad A, Perez-Cerda F. 2007a. Excitotoxic damage to white matter. Journal of anatomy 210(6):693-702.

Matute C, Sánchez-Gómez MV, Martinez-Millan L, Miledi R. 1997. Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. Proceedings of the National Academy of Sciences of the United States of America 94(16):8830-5.

Matute C, Torre I, Perez-Cerda F, Perez-Samartin A, Alberdi E, Etxebarria E, Arranz AM, Ravid R, Rodriguez-Antiguedad A, Sánchez-Gómez M and others. 2007b. P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. The Journal of neuroscience : the official journal of the Society for Neuroscience 27(35):9525-33.

May-Benson TA, Koomar JA, Teasdale A. 2009. Incidence of pre-, peri-, and post-natal birth and developmental problems of children with sensory processing disorder and children with autism spectrum disorder. Frontiers in integrative neuroscience 3:31.

Mayer M, Bhakoo K, Noble M. 1994. Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. Development 120(1):143-53.

McCarthy KD, de Vellis J. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. The Journal of cell biology 85(3):890-902.

McDonald JW, Shapiro SM, Silverstein FS, Johnston MV. 1998. Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. Experimental neurology 150(1):21-9.

McGrath J, Saha S, Chant D, Welham J. 2008. Schizophrenia: a concise overview of incidence, prevalence, and mortality. Epidemiologic reviews 30:67-76.

McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ and others. 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. The EMBO journal 15(20):5647-58.

McKinnon RD, Matsui T, Dubois-Dalcq M, Aaronson SA. 1990. FGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron 5(5):603-14.

McKinnon RD, Piras G, Ida JA, Jr., Dubois-Dalcq M. 1993. A role for TGF-beta in oligodendrocyte differentiation. The Journal of cell biology 121(6):1397-407.

McLaurin JA, Yong VW. 1995. Oligodendrocytes and myelin. Neurologic clinics 13(1):23-49.

McMorris FA, Furlanetto RW, Mozell RL, Carson MJ, Raible DW. 1990. Regulation of oligodendrocyte development by insulin-like growth factors and cyclic nucleotides. Annals of the New York Academy of Sciences 605:101-9.

McTigue DM, Horner PJ, Stokes BT, Gage FH. 1998. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. The Journal of neuroscience: the official journal of the Society for Neuroscience 18(14):5354-65.

McTigue DM, Tripathi RB. 2008. The life, death, and replacement of oligodendrocytes in the adult CNS. Journal of neurochemistry 107(1):1-19.

Medana I, Martinic MA, Wekerle H, Neumann H. 2001. Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. The American journal of pathology 159(3):809-15.

Merrill JE, Scolding NJ. 1999. Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. Neuropathology and applied neurobiology 25(6):435-58.

Metea MR, Newman EA. 2006. Glial cells dilate and constrict blood vessels: a mechanism of neurovascular coupling. The Journal of neuroscience: the official journal of the Society for Neuroscience 26(11):2862-70.

Mi S, Miller RH, Lee X, Scott ML, Shulag-Morskaya S, Shao Z, Chang J, Thill G, Levesque M, Zhang M and others. 2005. LINGO-1 negatively regulates myelination by oligodendrocytes. Nature neuroscience 8(6):745-51.

Mi S, Miller RH, Tang W, Lee X, Hu B, Wu W, Zhang Y, Shields CB, Miklasz S, Shea D and others. 2009. Promotion of central nervous system remyelination by induced differentiation of oligodendrocyte precursor cells. Annals of neurology 65(3):304-15.

Micu I, Jiang Q, Coderre E, Ridsdale A, Zhang L, Woulfe J, Yin X, Trapp BD, McRory JE, Rehak R and others. 2006. NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. Nature 439(7079):988-92.

Miller RH. 2002. Regulation of oligodendrocyte development in the vertebrate CNS. Progress in neurobiology 67(6):451-67.

Miyaoka T, Seno H, Itoga M, Iijima M, Inagaki T, Horiguchi J. 2000. Schizophrenia-associated idiopathic unconjugated hyperbilirubinemia (Gilbert's syndrome). The Journal of clinical psychiatry 61(11):868-71.

Moghaddam B, Adams BW. 1998. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. Science 281(5381):1349-52.

Monier A, Adle-Biassette H, Delezoide AL, Evrard P, Gressens P, Verney C. 2007. Entry and distribution of microglial cells in human embryonic and fetal cerebral cortex. Journal of neuropathology and experimental neurology 66(5):372-82.

Monif M, Reid CA, Powell KL, Smart ML, Williams DA. 2009. The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. The Journal of neuroscience : the official journal of the Society for Neuroscience 29(12):3781-91.

Mullins RD, Heuser JA, Pollard TD. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proceedings of the National Academy of Sciences of the United States of America 95(11):6181-6.

Naeye RL. 1978. Amniotic fluid infections, neonatal hyperbilirubinemia, and psychomotor impairment. Pediatrics 62(4):497-503.

Nagy JI, Rash JE. 2000. Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS. Brain research Brain research reviews 32(1):29-44.

Nair A, Frederick TJ, Miller SD. 2008. Astrocytes in multiple sclerosis: a product of their environment. Cell Molecular Life Sciences 65(17):2702-20.

Nave KA. 2010. Myelination and the trophic support of long axons. Nature reviews Neuroscience 11(4):275-83.

Neumann H, Medana IM, Bauer J, Lassmann H. 2002. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. Trends in neurosciences 25(6):313-9.

Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308(5726):1314-8.

Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB. 1996. Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. Journal of neuroscience research 43(3):299-314.

Noble M, Murray K, Stroobant P, Waterfield MD, Riddle P. 1988. Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature 333(6173):560-2.

Notarianni LJ. 1990. Plasma protein binding of drugs in pregnancy and in neonates. Clinical pharmacokinetics 18(1):20-36.

O'Connor KC, Appel H, Bregoli L, Call ME, Catz I, Chan JA, Moore NH, Warren KG, Wong SJ, Hafler DA and others. 2005. Antibodies from inflamed central nervous system tissue recognize myelin oligodendrocyte glycoprotein. Journal of immunology 175(3):1974-82.

Olney JW, Newcomer JW, Farber NB. 1999. NMDA receptor hypofunction model of schizophrenia. Journal of psychiatric research 33(6):523-33.

Onishi S, Kawade N, Itoh S, Isobe K, Sugiyama S. 1979. Postnatal development of uridine diphosphate glucuronyltransferase activity towards bilirubin and 2-aminophenol in human liver. The Biochemical journal 184(3):705-7.

Ontaneda D, Rae-Grant AD. 2009. Management of acute exacerbations in multiple sclerosis. Annals of Indian Academy of Neurology 12(4):264-72.

Orthmann-Murphy JL, Abrams CK, Scherer SS. 2008. Gap junctions couple astrocytes and oligodendrocytes. Journal of molecular neuroscience : MN 35(1):101-16.

Ostrow JD, Pascolo L, Tiribelli C. 2002. Mechanisms of bilirubin neurotoxicity. Hepatology 35(5):1277-80.

Othman T, Yan H, Rivkees SA. 2003. Oligodendrocytes express functional A1 adenosine receptors that stimulate cellular migration. Glia 44(2):166-72.

Palmela I, Cardoso FL, Bernas M, Correia L, Vaz AR, Silva RFM, Fernandes A, Kim KS, Brites D, Brito MA. 2011. Elevated levels of bilirubin and long-term exposure impair human brain microvascular endothelial cell integrity. Current neurovascular research 8(2):153-69.

Palmela I, Sasaki H, Cardoso FL, Moutinho M, Kim KS, Brites D, Brito MA. 2012. Time-dependent dual effects of high levels of unconjugated bilirubin on the human blood-brain barrier lining. Frontiers in cellular neuroscience 6:22.

Pang Y, Cai Z, Rhodes PG. 2005. Effect of tumor necrosis factor-alpha on developing optic nerve oligodendrocytes in culture. Journal of neuroscience research 80(2):226-34.

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF, Jr., Spray DC, Reichenbach A, Pannicke T and others. 2012. Glial cells in (patho)physiology. Journal of neurochemistry 121(1):4-27.

Pastor A, Chvatal A, Sykova E, Kettenmann H. 1995. Glycine- and GABA-activated currents in identified glial cells of the developing rat spinal cord slice. The European journal of neuroscience 7(6):1188-98.

Paszty K, Verma AK, Padanyi R, Filoteo AG, Penniston JT, Enyedi A. 2002. Plasma membrane Ca2+ATPase isoform 4b is cleaved and activated by caspase-3 during the early phase of apoptosis. The Journal of biological chemistry 277(9):6822-9.

Patneau DK, Wright PW, Winters C, Mayer ML, Gallo V. 1994. Glial cells of the oligodendrocyte lineage express both kainate- and AMPA-preferring subtypes of glutamate receptor. Neuron 12(2):357-71.

Paus T, Collins DL, Evans AC, Leonard G, Pike B, Zijdenbos A. 2001. Maturation of white matter in the human brain: a review of magnetic resonance studies. Brain research bulletin 54(3):255-66.

Perry VH, Nicoll JA, Holmes C. 2010. Microglia in neurodegenerative disease. Nature reviews Neurology 6(4):193-201.

Pette M, Fujita K, Wilkinson D, Altmann DM, Trowsdale J, Giegerich G, Hinkkanen A, Epplen JT, Kappos L, Wekerle H. 1990. Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. Proceedings of the National Academy of Sciences of the United States of America 87(20):7968-72.

Pfeiffer SE, Warrington AE, Bansal R. 1993. The oligodendrocyte and its many cellular processes. Trends in cell biology 3(6):191-7.

Pollard TD. 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. Annual review of biophysics and biomolecular structure 36:451-77.

Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG. 2005. Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. The Journal of biological chemistry 280(8):6447-54.

Pringle NP, Mudhar HS, Collarini EJ, Richardson WD. 1992. PDGF receptors in the rat CNS: during late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. Development 115(2):535-51.

Pugliatti M, Sotgiu S, Rosati G. 2002. The worldwide prevalence of multiple sclerosis. Clinical neurology and neurosurgery 104(3):182-91.

Raff MC, Lillien LE, Richardson WD, Burne JF, Noble MD. 1988. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature 333(6173):562-5.

Raff MC, Mirsky R, Fields KL, Lisak RP, Dorfman SH, Silberberg DH, Gregson NA, Leibowitz S, Kennedy MC. 1978. Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. Nature 274(5673):813-6.

Ransohoff RM, Perry VH. 2009. Microglial physiology: unique stimuli, specialized responses. Annual review of immunology 27:119-45.

Rejdak K, Jackson S, Giovannoni G. 2010. Multiple sclerosis: a practical overview for clinicians. British medical bulletin 95:79-104.

Reynolds R, Wilkin GP. 1988. Development of macroglial cells in rat cerebellum. II. An in situ immunohistochemical study of oligodendroglial lineage from precursor to mature myelinating cell. Development 102(2):409-25.

Rezaie P, Dean A. 2002. Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. Neuropathology: official journal of the Japanese Society of Neuropathology 22(3):106-32.

Richardson WD, Pringle N, Mosley MJ, Westermark B, Dubois-Dalcq M. 1988. A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. Cell 53(2):309-19.

Riento K, Ridley AJ. 2003. Rocks: multifunctional kinases in cell behaviour. Nature reviews Molecular cell biology 4(6):446-56.

Robinson S, Tani M, Strieter RM, Ransohoff RM, Miller RH. 1998. The chemokine growth-regulated oncogene-alpha promotes spinal cord oligodendrocyte precursor proliferation. The Journal of neuroscience: the official journal of the Society for Neuroscience 18(24):10457-63.

Rodrigues CM, Sola S, Brites D. 2002a. Bilirubin induces apoptosis via the mitochondrial pathway in developing rat brain neurons. Hepatology 35(5):1186-95.

Rodrigues CM, Sola S, Castro RE, Laires PA, Brites D, Moura JJ. 2002b. Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis. Journal of lipid research 43(6):885-94.

Ron D, Walter P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews Molecular cell biology 8(7):519-29.

Rosenberg PA, Dai W, Gan XD, Ali S, Fu J, Back SA, Sanchez RM, Segal MM, Follett PL, Jensen FE and others. 2003. Mature myelin basic protein-expressing oligodendrocytes are insensitive to kainate toxicity. Journal of neuroscience research 71(2):237-45.

Rowitch DH. 2004. Glial specification in the vertebrate neural tube. Nature reviews Neuroscience 5(5):409-19.

Rubin RA, Balow B, Fisch RO. 1979. Neonatal serum bilirubin levels related to cognitive development at ages 4 through 7 years. The Journal of pediatrics 94(4):601-4.

Rutishauser U, Landmesser L. 1996. Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. Trends in neurosciences 19(10):422-7.

Salamy A, Eldredge L, Tooley WH. 1989. Neonatal status and hearing loss in high-risk infants. The Journal of pediatrics 114(5):847-52.

Salinska E, Danysz W, Lazarewicz JW. 2005. The role of excitotoxicity in neurodegeneration. Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences 43(4):322-39.

Salter MG, Fern R. 2005. NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. Nature 438(7071):1167-71.

Sánchez-Gómez MV, Alberdi E, Ibarretxe G, Torre I, Matute C. 2003. Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors. The Journal of neuroscience: the official journal of the Society for Neuroscience 23(29):9519-28.

Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B. 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74(4):691-705.

Schousboe A. 2003. Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. Neurochemical research 28(2):347-52.

Schroder M, Kaufman RJ. 2005. The mammalian unfolded protein response. Annual review of biochemistry 74:739-89.

Scolding NJ, Frith S, Linington C, Morgan BP, Campbell AK, Compston DA. 1989. Myelin-oligodendrocyte glycoprotein (MOG) is a surface marker of oligodendrocyte maturation. Journal of neuroimmunology 22(3):169-76.

Scurlock B, Dawson G. 1999. Differential responses of oligodendrocytes to tumor necrosis factor and other pro-apoptotic agents: role of ceramide in apoptosis. Journal of neuroscience research 55(4):514-22.

Seidman DS, Paz I, Stevenson DK, Laor A, Danon YL, Gale R. 1991. Neonatal hyperbilirubinemia and physical and cognitive performance at 17 years of age. Pediatrics 88(4):828-33.

Seifert G, Schilling K, Steinhauser C. 2006. Astrocyte dysfunction in neurological disorders: a molecular perspective. Nature reviews Neuroscience 7(3):194-206.

Seki T, Arai Y. 1991. The persistent expression of a highly polysialylated NCAM in the dentate gyrus of the adult rat. Neuroscience research 12(4):503-13.

Seki T, Arai Y. 1993. Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neuroscience research 17(4):265-90.

Sequeira D, Watchko JF, Daood MJ, O'Day TL, Mahmood B. 2007. Unconjugated bilirubin efflux by bovine brain microvascular endothelial cells in vitro. Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies 8(6):570-5.

Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A and others. 2004. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21(1):81-93.

Shapiro SM. 2003. Bilirubin toxicity in the developing nervous system. Pediatric neurology 29(5):410-21.

Shapiro SM. 2005. Definition of the clinical spectrum of kernicterus and bilirubin-induced neurologic dysfunction (BIND). Journal of perinatology: official journal of the California Perinatal Association 25(1):54-9.

Shapiro SM. 2010. Chronic bilirubin encephalopathy: diagnosis and outcome. Seminars in fetal & neonatal medicine 15(3):157-63.

Shi HB, Kakazu Y, Shibata S, Matsumoto N, Nakagawa T, Komune S. 2006. Bilirubin potentiates inhibitory synaptic transmission in lateral superior olive neurons of the rat. Neuroscience research 55(2):161-70.

Silberberg DH, Schutta HS. 1967. The effects of unconjugated bilirubin and related pigments on cultures of rat cerebellum. Journal of neuropathology and experimental neurology 26(4):572-83.

Silva RFM, Mata LR, Gulbenkian S, Brito MA, Tiribelli C, Brites D. 1999. Inhibition of glutamate uptake by unconjugated bilirubin in cultured cortical rat astrocytes: role of concentration and pH. Biochemical and biophysical research communications 265(1):67-72.

Silva RFM, Rodrigues CM, Brites D. 2002. Rat cultured neuronal and glial cells respond differently to toxicity of unconjugated bilirubin. Pediatric research 51(4):535-41.

Silva SL, Osorio C, Vaz AR, Barateiro A, Falcão AS, Silva RFM, Brites D. 2011. Dynamics of neuron-glia interplay upon exposure to unconjugated bilirubin. Journal of neurochemistry 117(3):412-24.

Silva SL, Vaz AR, Barateiro A, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2010. Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. Neurobiology of disease 40(3):663-75.

Silva SL, Vaz AR, Diogenes MJ, van Rooijen N, Sebastiao AM, Fernandes A, Silva RFM, Brites D. 2012. Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia, and prevented by glycoursodeoxycholic acid and interleukin-10. Neuropharmacology 62(7):2398-408.

Simons M, Trajkovic K. 2006. Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. Journal of cell science 119(Pt 21):4381-9.

Simpson EH, Kellendonk C, Kandel E. 2010. A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. Neuron 65(5):585-96.

Simpson PB, Armstrong RC. 1999. Intracellular signals and cytoskeletal elements involved in oligodendrocyte progenitor migration. Glia 26(1):22-35.

Sloane JA, Vartanian TK. 2007. WAVE1 and regulation of actin nucleation in myelination. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 13(5):486-91.

Smiley JF, Rosoklija G, Mancevski B, Pergolizzi D, Figarsky K, Bleiwas C, Duma A, Mann JJ, Javitt DC, Dwork AJ. 2011. Hemispheric comparisons of neuron density in the planum temporale of schizophrenia and nonpsychiatric brains. Psychiatry research 192(1):1-11.

Smith KJ, Lassmann H. 2002. The role of nitric oxide in multiple sclerosis. Lancet neurology 1(4):232-41.

Smith MJ, Wang L, Cronenwett W, Mamah D, Barch DM, Csernansky JG. 2011. Thalamic morphology in schizophrenia and schizoaffective disorder. Journal of psychiatric research 45(3):378-85.

Sommer I, Schachner M. 1981. Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Developmental biology 83(2):311-27.

Song H, Stevens CF, Gage FH. 2002. Astroglia induce neurogenesis from adult neural stem cells. Nature 417(6884):39-44.

Song J, Goetz BD, Baas PW, Duncan ID. 2001. Cytoskeletal reorganization during the formation of oligodendrocyte processes and branches. Molecular and cellular neurosciences 17(4):624-36.

Soorani-Lunsing I, Woltil HA, Hadders-Algra M. 2001. Are moderate degrees of hyperbilirubinemia in healthy term neonates really safe for the brain? Pediatric research 50(6):701-5.

Soulet D, Rivest S. 2008. Microglia. Current biology: CB 18(12):R506-8.

Spohr TC, Choi JW, Gardell SE, Herr DR, Rehen SK, Gomes FC, Chun J. 2008. Lysophosphatidic acid receptor-dependent secondary effects via astrocytes promote neuronal differentiation. The Journal of biological chemistry 283(12):7470-9.

Sriburi R, Jackowski S, Mori K, Brewer JW. 2004. XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. The Journal of cell biology 167(1):35-41.

Stark AK, Uylings HB, Sanz-Arigita E, Pakkenberg B. 2004. Glial cell loss in the anterior cingulate cortex, a subregion of the prefrontal cortex, in subjects with schizophrenia. The American journal of psychiatry 161(5):882-8.

Steelman AJ, Li J. 2011. Poly(I:C) promotes TNFalpha/TNFR1-dependent oligodendrocyte death in mixed glial cultures. Journal of neuroinflammation 8:89.

Stephan KE, Baldeweg T, Friston KJ. 2006. Synaptic plasticity and dysconnection in schizophrenia. Biological psychiatry 59(10):929-39.

Stephan KE, Friston KJ, Frith CD. 2009. Dysconnection in schizophrenia: from abnormal synaptic plasticity to failures of self-monitoring. Schizophrenia bulletin 35(3):509-27.

Stevens B, Porta S, Haak LL, Gallo V, Fields RD. 2002. Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. Neuron 36(5):855-68.

Stevenson DK, Vreman HJ, Wong RJ, Contag CH. 2001. Carbon monoxide and bilirubin production in neonates. Seminars in perinatology 25(2):85-93.

Stolt CC, Lommes P, Friedrich RP, Wegner M. 2004. Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. Development 131(10):2349-58.

Stolt CC, Schlierf A, Lommes P, Hillgartner S, Werner T, Kosian T, Sock E, Kessaris N, Richardson WD, Lefebvre V and others. 2006. SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. Developmental cell 11(5):697-709.

Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nervous tissue. Journal of neuroscience methods 37(2):173-82.

Stradal TE, Rottner K, Disanza A, Confalonieri S, Innocenti M, Scita G. 2004. Regulation of actin dynamics by WASP and WAVE family proteins. Trends in cell biology 14(6):303-11.

Sun T, Pringle NP, Hardy AP, Richardson WD, Smith HK. 1998. Pax6 influences the time and site of origin of glial precursors in the ventral neural tube. Molecular and cellular neurosciences 12(4-5):228-39.

Svitkina TM, Borisy GG. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. The Journal of cell biology 145(5):1009-26.

Swerdlow NR. 2010. Integrative circuit models and their implications for the pathophysiologies and treatments of the schizophrenias. Current topics in behavioral neurosciences 4:555-83.

Sykova E, Nicholson C. 2008. Diffusion in brain extracellular space. Physiological reviews 88(4):1277-340.

Symons M, Derry JM, Karlak B, Jiang S, Lemahieu V, McCormick F, Francke U, Abo A. 1996. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 84(5):723-34.

Szuchet S, Polak PE, Yim SH. 1986. Mature oligodendrocytes cultured in the absence of neurons recapitulate the ontogenic development of myelin membranes. Developmental neuroscience 8(4):208-21.

Takebayashi H, Nabeshima Y, Yoshida S, Chisaka O, Ikenaka K. 2002. The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Current biology : CB 12(13):1157-63.

Takenawa T, Miki H. 2001. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. Journal of cell science 114(Pt 10):1801-9.

Tallantyre EC, Bo L, Al-Rawashdeh O, Owens T, Polman CH, Lowe JS, Evangelou N. 2010. Clinico-pathological evidence that axonal loss underlies disability in progressive multiple sclerosis. Multiple sclerosis 16(4):406-11.

Tang DG, Tokumoto YM, Apperly JA, Lloyd AC, Raff MC. 2001. Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. Science 291(5505):868-71.

Terada N, Kidd GJ, Kinter M, Bjartmar C, Moran-Jones K, Trapp BD. 2005. Beta IV tubulin is selectively expressed by oligodendrocytes in the central nervous system. Glia 50(3):212-22.

Thaker GK. 2008. Neurophysiological endophenotypes across bipolar and schizophrenia psychosis. Schizophrenia bulletin 34(4):760-73.

Theodosis DT, El Majdoubi M, Pierre K, Poulain DA. 1998. Factors governing activity-dependent structural plasticity of the hypothalamoneurohypophysial system. Cellular and molecular neurobiology 18(2):285-98.

Theodosis DT, Poulain DA, Oliet SH. 2008. Activity-dependent structural and functional plasticity of astrocyte-neuron interactions. Physiological reviews 88(3):983-1008.

Thomson CE, Hunter AM, Griffiths IR, Edgar JM, McCulloch MC. 2006. Murine spinal cord explants: a model for evaluating axonal growth and myelination in vitro. Journal of neuroscience research 84(8):1703-15.

Thorburne SK, Juurlink BH. 1996. Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. Journal of neurochemistry 67(3):1014-22.

Timsit S, Martinez S, Allinquant B, Peyron F, Puelles L, Zalc B. 1995. Oligodendrocytes originate in a restricted zone of the embryonic ventral neural tube defined by DM-20 mRNA expression. The Journal of neuroscience: the official journal of the Society for Neuroscience 15(2):1012-24.

Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH, Bahn S. 2003. Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet 362(9386):798-805.

Todd DJ, Lee AH, Glimcher LH. 2008. The endoplasmic reticulum stress response in immunity and autoimmunity. Nature Reviews Immunology 8(9);663-74.

Todorich B, Pasquini JM, Garcia CI, Paez PM, Connor JR. 2009. Oligodendrocytes and myelination: the role of iron. Glia 57(5):467-78.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. 1998. Axonal transection in the lesions of multiple sclerosis. The New England journal of medicine 338(5):278-85.

Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, Geertman R, Ransohoff RM, Miller RH. 2002. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. Cell 110(3):373-83.

Tsai HH, Tessier-Lavigne M, Miller RH. 2003. Netrin 1 mediates spinal cord oligodendrocyte precursor dispersal. Development 130(10):2095-105.

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287(5453):664-6.

Uranova NA, Orlovskaya D, Vikhreva O, Zimina I, Kolomeets N, Vostrikov V, Rachmanova V. 2001. Electron microscopy of oligodendroglia in severe mental illness. Brain research bulletin 55(5):597-610.

Uranova NA, Vostrikov VM, Vikhreva OV, Zimina IS, Kolomeets NS, Orlovskaya DD. 2007. The role of oligodendrocyte pathology in schizophrenia. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 10(4):537-45.

Valerio A, Ferrario M, Dreano M, Garotta G, Spano P, Pizzi M. 2002. Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells. Molecular and cellular neurosciences 21(4):602-15.

Valli A, Sette A, Kappos L, Oseroff C, Sidney J, Miescher G, Hochberger M, Albert ED, Adorini L. 1993. Binding of myelin basic protein peptides to human histocompatibility leukocyte antigen class II molecules and their recognition by T cells from multiple sclerosis patients. The Journal of clinical investigation 91(2):616-28.

Vallstedt A, Klos JM, Ericson J. 2005. Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. Neuron 45(1):55-67.

Vattem KM, Wek RC. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 101(31):11269-74.

Vaz AR, Delgado-Esteban M, Brito MA, Bolanos JP, Brites D, Almeida A. 2010. Bilirubin selectively inhibits cytochrome c oxidase activity and induces apoptosis in immature cortical neurons: assessment of the protective effects of glycoursodeoxycholic acid. Journal of neurochemistry 112(1):56-65.

Vaz AR, Silva SL, Barateiro A, Fernandes A, Falcão AS, Brito MA, Brites D. 2011. Pro-inflammatory cytokines intensify the activation of NO/NOS, JNK1/2 and caspase cascades in immature neurons exposed to elevated levels of unconjugated bilirubin. Experimental neurology 229(2):381-90.

Vela JM, Molina-Holgado E, Arevalo-Martin A, Almazan G, Guaza C. 2002. Interleukin-1 regulates proliferation and differentiation of oligodendrocyte progenitor cells. Molecular and cellular neurosciences 20(3):489-502.

Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS, Foster AC. 2004. Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. The European journal of neuroscience 20(5):1150-60.

Verkhratsky A. 2005. Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. Physiological reviews 85(1):201-79.

Verkhratsky A, Orkand RK, Kettenmann H. 1998. Glial calcium: homeostasis and signaling function. Physiological reviews 78(1):99-141.

Verma G, Datta M. 2012. The critical role of JNK in the ER-mitochondrial crosstalk during apoptotic cell death. Journal of cellular physiology 227(5):1791-5.

Verney C, Monier A, Fallet-Bianco C, Gressens P. 2010. Early microglial colonization of the human forebrain and possible involvement in periventricular white-matter injury of preterm infants. Journal of anatomy 217(4):436-48.

Villa PG, Henzel WJ, Sensenbrenner M, Henderson CE, Pettmann B. 1998. Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. Journal of cell science 111 (Pt 6):713-22.

Volk DW, Lewis DA. 2010. Prefrontal cortical circuits in schizophrenia. Current topics in behavioral neurosciences 4:485-508.

Voyvodic JT. 1989. Target size regulates calibre and myelination of sympathetic axons. Nature 342(6248):430-3.

Vreman HJ, Wong RJ, Stevenson DK. 2004. Phototherapy: current methods and future directions. Seminars in perinatology 28(5):326-33.

Wallraff A, Kohling R, Heinemann U, Theis M, Willecke K, Steinhauser C. 2006. The impact of astrocytic gap junctional coupling on potassium buffering in the hippocampus. The Journal of neuroscience: the official journal of the Society for Neuroscience 26(20):5438-47.

Walters SN, Morell P. 1981. Effects of altered thyroid states on myelinogenesis. Journal of neurochemistry 36(5):1792-801.

Wang Y, Tang BL. 2006. SNAREs in neurons - beyond synaptic vesicle exocytosis (Review). Molecular membrane biology 23(5):377-84.

Warf BC, Fok-Seang J, Miller RH. 1991. Evidence for the ventral origin of oligodendrocyte precursors in the rat spinal cord. The Journal of neuroscience: the official journal of the Society for Neuroscience 11(8):2477-88.

Watchko JF, Lin Z. 2010. Exploring the genetic architecture of neonatal hyperbilirubinemia. Seminars in fetal & neonatal medicine 15(3):169-75.

Watchko JF, Maisels MJ. 2003. Jaundice in low birthweight infants: pathobiology and outcome. Archives of disease in childhood Fetal and neonatal edition 88(6):F455-8.

Watchko JF, Oski FA. 1992. Kernicterus in preterm newborns: past, present, and future. Pediatrics 90(5):707-15.

Waxman SG. 2006. Axonal conduction and injury in multiple sclerosis: the role of sodium channels. Nature reviews Neuroscience 7(12):932-41.

Weaver AM, Young ME, Lee WL, Cooper JA. 2003. Integration of signals to the Arp2/3 complex. Current opinion in cell biology 15(1):23-30.

Werner P, Pitt D, Raine CS. 2001. Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. Annals of neurology 50(2):169-80.

White R, Gonsior C, Kramer-Albers EM, Stohr N, Huttelmaier S, Trotter J. 2008. Activation of oligodendroglial Fyn kinase enhances translation of mRNAs transported in hnRNP A2-dependent RNA granules. The Journal of cell biology 181(4):579-86.

Wiggins RC. 1982. Myelin development and nutritional insufficiency. Brain research 257(2):151-75.

Williamson AV, Mellor JR, Grant AL, Randall AD. 1998. Properties of GABA(A) receptors in cultured rat oligodendrocyte progenitor cells. Neuropharmacology 37(7):859-73.

Wong RJ, DeSandre GH, Sibley E, et al. 2006. Neonatal jaundice and liver disease. In: Martin RJ,

Fanaroff AA, Walsh MC, editors. Fanaroff and Martin's neonatal-perinatal medicine: diseases of the fetus and infant. 8th edition. Philadelphia:Mosby Elsevier:1419-65.

Woo TU, Crowell AL. 2005. Targeting synapses and myelin in the prevention of schizophrenia. Schizophrenia research 73(2-3):193-207.

Woodruff RH, Tekki-Kessaris N, Stiles CD, Rowitch DH, Richardson WD. 2001. Oligodendrocyte development in the spinal cord and telencephalon: common themes and new perspectives. International journal of developmental neuroscience: the official journal of the International Society for Developmental Neuroscience 19(4):379-85.

Wootla B, Eriguchi M, Rodriguez M. 2012. Is multiple sclerosis an autoimmune disease? Autoimmune diseases 2012:969657.

Xin M, Yue T, Ma Z, Wu FF, Gow A, Lu QR. 2005. Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 25(6):1354-65.

Yamashima T. 2004. Ca2+-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell calcium 36(3-4):285-93.

Yan H, Wood PM. 2000. NT-3 weakly stimulates proliferation of adult rat O1(-)O4(+) oligodendrocyte-lineage cells and increases oligodendrocyte myelination in vitro. Journal of neuroscience research 62(3):329-35.

Ye J, Rawson RB, Komuro R, Chen X, Dave UP, Prywes R, Brown MS, Goldstein JL. 2000. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Molecular cell 6(6):1355-64.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107(7):881-91.

Yu WP, Collarini EJ, Pringle NP, Richardson WD. 1994. Embryonic expression of myelin genes: evidence for a focal source of oligodendrocyte precursors in the ventricular zone of the neural tube. Neuron 12(6):1353-62.

Zalc B, Monge M, Dupouey P, Hauw JJ, Baumann NA. 1981. Immunohistochemical localization of galactosyl and sulfogalactosyl ceramide in the brain of the 30-day-old mouse. Brain research 211(2):341-54.

Zhang H, Jarjour AA, Boyd A, Williams A. 2011. Central nervous system remyelination in culture--a tool for multiple sclerosis research. Experimental neurology 230(1):138-48.

Zhang J, Markovic-Plese S, Lacet B, Raus J, Weiner HL, Hafler DA. 1994. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. The Journal of experimental medicine 179(3):973-84.

Zhang L, Liu W, Tanswell AK, Luo X. 2003. The effects of bilirubin on evoked potentials and long-term potentiation in rat hippocampus in vivo. Pediatric research 53(6):939-44.

Zhang SC. 2001. Defining glial cells during CNS development. Nature reviews Neuroscience 2(11):840-3.

Zhang X, Haaf M, Todorich B, Grosstephan E, Schieremberg H, Surguladze N, Connor JR. 2005. Cytokine toxicity to oligodendrocyte precursors is mediated by iron. Glia 52(3):199-208.

Zhao XH, Jin WL, Ju G. 2007. An in vitro study on the involvement of LINGO-1 and Rho GTPases in Nogo-A regulated differentiation of oligodendrocyte precursor cells. Molecular and cellular neurosciences 36(2):260-9.

Zhou Q, Anderson DJ. 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109(1):61-73.

Ziak D, Chvatal A, Sykova E. 1998. Glutamate-, kainate- and NMDA-evoked membrane currents in identified glial cells in rat spinal cord slice. Physiological research/Academia Scientiarum Bohemoslovaca 47(5):365-75.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes & development 12(7):982-95.

Chapter II

ER STRESS, MITOCHONDRIAL DYSFUNCTION AND CALPAIN/JNK ACTIVATION COOPERATE TO TRIGGER OLIGODENDROCYTE PRECURSOR CELL DEATH BY UNCONJUGATED BILIRUBIN

Andreia Barateiro[1], Ana R. Vaz[1], Sandra L. Silva[1], Adelaide Fernandes[1,2], Dora Brites[1,2]

[1]Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL),
Faculty of Pharmacy, University of Lisbon,
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

[2]Department of Biochemistry and Human Biology,
Faculty of Pharmacy, University of Lisbon
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

Neuromolecular Medicine (2012), *in press*.
[Epub ahead of print]

Acknowledgments

This work was supported by the strategic project PEst-OE/SAU/UI4013/2011 and PTDC/SAU-NEU/64385/2006 grants from Fundação para a Ciência e a Tecnologia), Lisbon, Portugal (to D. B.). A. B. was recipient of a PhD fellowship (SFRH/BD/43885/2008) from FCT. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Research on the mechanisms of bilirubin-induced neurologic dysfunction focuses mainly on neuronal death, astrocyte-mediated events and microglia activation. Although myelin damage by unconjugated bilirubin (UCB) has been documented in neonatal *kernicterus* cases, the events leading to myelination impairment were never explored. This condition may occur by reduced oligodendrocyte precursor cell (OPC) number or failure of OPC to differentiate in myelinating oligodendrocytes. We have shown that UCB elicits an inflammatory response, glutamate release and reactive oxygen species (ROS) generation in neurons and glial cells, biomolecules with toxic properties on OPC. Hence, we propose to examine whether UCB determines OPC demise and, if so, which signalling pathways are involved. Our results show that OPC display increased apoptosis and necrosis-like cell death upon UCB exposure, mediated by early signals of endoplasmic reticulum (ER) stress (e.g. upregulation of glucose-regulated protein (GRP) 78, inositol-requiring enzyme (IRE)-1α and activation transcription factor (ATF)-6, as well as activation of caspase-2 and c-Jun *N*-terminal kinase (JNK)), followed by mitochondrial dysfunction (e.g. loss of mitochondria membrane potential and caspase-9 activation). Later calpain activation points to intracellular Ca^{2+} overload and intervention of both ER and mitochondria. Downstream production of ROS may result from mitochondria damage and secondary injuries, possible determining the second cycle of GRP78, IRE-1α, caspase-2 and JNK activation. Moreover, inhibition of caspases, calpains and oxidative stress, by using specific inhibitors, prevented UCB-induced OPC death. UCB did not induce the release of cytokines or glutamate by OPC. These results indicate that UCB by reducing OPC survival, through a cascade of programmed intracellular events triggered by ER stress and mitochondrial dysfunction, can compromise myelinogenesis.

Keywords: Cell death; ER-mitochondria unit disruption; Oligodendrocyte precursor primary cultures; Unconjugated bilirubin toxicity.

1. Introduction

Elevated levels of unconjugated bilirubin (UCB) are common in neonatal life. Some infants evidence susceptibility to bilirubin-induced neurologic dysfunction (BIND), mainly if premature (Lauer and Spector 2011). Even in term infants that underwent double volume exchange transfusion due to high total serum bilirubin levels, acute encephalopathy revealed to determine abnormal neurodevelopmental outcome (Mukhopadhyay et al. 2010). Devastating effects are more frequent if hyperbilirubinemia occurs within the 1st postnatal week, indicating an early brain development window of vulnerability to BIND. Chronic bilirubin encephalopathy, referred as *kernicterus*, is the pathological term used for the yellow staining of the deep nuclei of the brain (Shapiro 2010). Brain sections of icteric infants showed UCB within neurons, neuronal processes, and microglia (Martich-Kriss et al. 1995; Hansen 2000). Interestingly, recent histopathological findings from a premature infant with *kernicterus* showed a decrease in the density of myelinated fibers and a loss of axons in the cerebellar sections (Brito et al. 2011), suggesting altered myelination.

Several years ago, Silberberg and Schutta (1967) reported that UCB adversely affects myelination in tissue cultures of rat cerebellum while no discernible change was produced by the inactive photo-oxidized compound. Binding of UCB to myelin basic protein (MBP) was later evidenced by difference spectra and circular dichroism spectrum (Gurba and Zand 1974). These findings were corroborated by the high concentrations of UCB found in the myelin fraction after UCB injection into rats (Hansen et al. 2001) and by magnetic resonance imaging scans revealing reduction of white matter volume and delay in hemispheric myelination in infants with severe UCB encephalopathy outcomes (Gkoltsiou et al. 2008). In fact, cerebellum was evidenced to be the most often pigmented region of the brain after basal ganglia, revealing marked loss of neurons, demyelination, and prominent gliosis (Ahdab-Barmada and Moossy 1984; Claireaux 2003).

Interestingly, although several cytototoxic effects have been demonstrated in neurons (Silva et al. 2001; Fernandes and Brites 2009), astrocytes (Silva et al. 1999; Fernandes et al. 2004) and microglia (Gordo et al. 2006; Silva et al. 2010), only one study demonstrated that high levels of UCB decrease the viability of mature oligodendrocyte (OL), via the induction of nitric oxide (NO) production (Genc et al. 2003). However, UCB was shown to induce other glial reactivity and the release of pro-inflammatory cytokines by astrocytes and microglia (Fernandes et al. 2007; Fernandes et al. 2011; Silva et al. 2010). These cytokines showed to have cumulative effects on the activation of NO synthase and caspase cascades in neurons (Vaz et al. 2011b).

Our previous work evidenced that UCB triggers oxidative stress in neocortical synaptosomes (Brito et al. 2004) and more markedly affected neurons than astrocytes (Brito et al. 2008a; Brito et al. 2008b). Immature neurons showed to be more prone to UCB damage than differentiated cells in culture (Falcão et al. 2006). Intracellular pathways leading to apoptosis of immature neurons involve a bioenergetic and oxidative crisis with mitochondria dysfunction and activation of caspase-9 and -3 (Vaz et al. 2010). In addition, UCB-induced damage to immature neurons also showed to include upregulation of neuronal NO synthase expression and production of NO through overstimulation of glutamate receptors, thus indicating accumulation of extracellular glutamate and excitotoxicity as key determinants in BIND (Vaz et al. 2011b).

Once both immature neurons and astrocytes (Falcão et al. 2005, 2006) showed increased vulnerability to UCB injury, as compared to more differentiated cells, we wondered whether survival of oligodendrocyte precursor cells (OPC), the cells that suffer differentiation after axonal contact in order to myelinate, could be compromised when exposed to UCB, a feature never investigated before.

Mature OL arise from OPC that proliferate and differentiate just before and after birth. In the central nervous system, OPC develop according to a highly regulated program of migration, proliferation, differentiation and myelination (Pfeiffer et al. 1993). During this progress along a well-established lineage, which is highly correlated *in vitro* and *in vivo* (Pfeiffer et al. 1993; Miller 2002), OPC acquire complex morphological features and express specific markers (Levine et al. 2001). Initially, OPC present a bipolar morphology, and express the NG2 proteoglycan and a ganglioside recognized by the A2B5 antibody (Gard and Pfeiffer 1990; Nishiyama et al. 1996). The presence of both fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor (PDGF) stimulate proliferation and inhibit terminal differentiation (Bogler et al. 1990). A multiprocessed phenotype and expression of myelin components, such as MBP, are acquired along cell differentiation and maturation (Monge et al. 1986).

Once mature OL and OPC are very susceptible to reactive oxygen species (ROS), inflammatory cytokines and glutamate excitotoxicity (McTigue and Tripathi 2008), as well as to perturbations in intracellular Ca^{2+} (Fields and Stevens-Graham 2002), we hypothesized that UCB would interfere in OPC survival. Excessive cytosolic Ca^{2+} accumulation, observed in experimental *in vitro* studies with UCB (Brito et al., 2004), leads to ROS production and release of proapoptotic factors (Khorchid et al. 2002), besides activating calpains, phospholipases, endonucleases and NO synthase (Benarroch 2009). Perturbations in Ca^{2+} homeostasis can also interfere with oxidative protein folding, leading to endoplasmic reticulum (ER) stress (Rutkowski and Kaufman

2004). The accumulation of unfolded proteins in the ER lumen can induce the transcription of genes encoding ER resident chaperones. Signalling molecules, double stranded ribonucleic acid-activated protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring kinase 1α (IRE-1α) and activating transcription factor 6 (ATF-6) sense the presence of unfolded proteins in the ER lumen and transduce signals to the cytoplasm and the nucleus (Rutkowski and Kaufman 2004; Li and Lee 2006). Moreover, during the accumulation of misfolded proteins, glucose-regulated protein (GRP) 78 and 94 are upregulated (Ruiz et al. 2010) and dissociate from the three ER transmembrane signalling proteins PERK, IRE-1α and ATF-6, which leads to their activation, and triggers the unfolded protein response (Kulkarni et al. 2011; Wu and Kaufman 2006). The dissociated IRE-1α is phosphorylated (Kim et al. 2006), interacts with the adaptor protein tumor necrosis factor receptor-associated factor-2 and triggers c-Jun *N*-terminal kinase (JNK) activation (Verma and Datta 2011). Cytosolic Ca^{2+} overload is, therefore, linked to ER stress, mitochondrial dysfunction and OPC apoptosis. Nevertheless, these intracellular pathways and subcellular compartments were never investigated as targets of UCB injury to OPC, which similarly to other stem/precursor cells are highly responsive to environmental stimuli (Kotter et al. 2011). In this study, we have evaluated the cytotoxic effects of UCB on OPC and the pathways involved in the cell death process. Our data indicate that loss of OPC viability by UCB is due to both apoptosis and necrosis-like cell death, resulting from ER stress, mitochondrial dysfunction, ROS production, as well as activation of caspase-2, -9 and -3, calpains and JNK. Collectively, these events suggest that UCB may compromise oligodendrogenesis, myelination and repair, thus predisposing to BIND.

2. Material and Methods

2.1. Animals

Wistar rats were maintained on a 12 h light/dark cycle under conditions of constant temperature and humidity. Animals were supplied with standard laboratory chow and water ad libitum. Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

2.2. Primary culture of OPC

Primary OPC cultures were prepared from rat neonatal cortices according to the method of Chen and collaborators (Chen et al. 2007), with minor modifications. Briefly, a mixed glial culture isolated from neonatal Wistar rat was grown for 10 days in Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 20% fetal bovine serum (Biochrom AG), 1 mM sodium pyruvate (Biochrom AG), 2 mM L-glutamine (Biochrom AG) and 0.5% antibiotic antimycotic (Sigma Chemical Co, St. Louis, MO, USA). OPC, isolated by shaking, were cultured in defined medium containing serum-free DMEM, L-glutamine (4 mM), sodium pyruvate (1 mM), bovine serum albumin (0.1%) (Sigma), apo-transferrin (50 µg/ml) (Sigma), insulin (5 µg/ml) (Sigma), sodium selenite (30 nM) (Sigma), D-biotin (10 nM) (Sigma), hydrocortisone (10 nM) (Sigma), human PDGF-AA (30 ng/ml) (PeproTech, Rocky Hill, NJ, USA) and basic FGF-2 (30 ng/ml) (PeproTech). After 6 days *in vitro* (DIV), cell purity and phenotypic characteristics were routinely assessed by double immunocytochemical staining using antibodies directed to NG2 (#AB5320, Millipore, Billerica, MA, USA) to identify the OPC, nestin (#MAB353, Millipore) for immature cells, A2B5 (#MAB312, Millipore) for preoligodendrocytes or MBP (#AB980, Millipore) for mature OL. Possible contaminating glial cells were assessed by using goat anti-ionized calcium-binding adaptor molecule 1 (Iba-1) (#ab5076, AbCam Cambridge, UK) for microglia and glial fibrillary acidic protein (GFAP) (#GFAP-GA5, Novocastra, Wetzlar, Germany) for astrocytes.

2.3. OPC treatment

OPC cultures were incubated with 50 µM UCB (Sigma) plus 100 µM human serum albumin (HSA) (fraction V, fatty acid free, Sigma), or with 100 µM HSA (vehicle). UCB was purified according to the method of McDonagh (McDonagh and Assisi 1972). All UCB solutions were extemporarily prepared from 10 mM stock solution in 0.1 M NaOH, and the pH adjusted to 7.4 by addition of equal amounts of 0.1 M HCl. Preparation of solutions and execution of experiments were performed under light protection (vial wrapped in tin foil and dim light) to avoid UCB photodegradation. In order to mimic clinically relevant conditions, a molar ratio of UCB to HSA of 0.5 was used (50 µM UCB:100 µM HSA). By consistently using the same experimental model, besides having suitable and stable UCB solutions, we can compare susceptibilities between different nerve cells to UCB.

In parallel studies, OPC were incubated with 1 µM z-VAD-FMK (Enzo Life Sciences, Farmingdale, New York, US), a caspase inhibitor, with 5 µM calpeptin

(Tocris Bioscience, Ellisville, Missouri, USA), a calpain inhibitor, or with 100 μM N-acetylcysteine (NAC) (Sigma), a precursor of glutathione synthesis described as antioxidant, alone or in combination with UCB.

2.4. Cell death assays

Evaluation of apoptotic cells was performed using TUNEL assay (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells plated on coverslips were fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate in PBS for 2 min on ice. After rinsing in PBS, the coverslips were incubated with TUNEL reaction mixture for 60 min at 37ºC in the absence of light. The cells were rinsed, stained with Hoechst 33258 dye (Sigma) to visualize the nuclei and mounted. Green-fluorescence and U.V. images of fifteen random microscopic fields (original magnification: 400x) were acquired per sample using a fluorescence microscope (Axioskope®, Zeiss, Germany). The percentage of TUNEL-positive cells emitting green fluorescence and the total number of cells were counted by investigators blinded as to the treatment received by cells and expressed as fold vs. vehicle-treated cells.

Necrotic-like cell death was evaluated by propidium iodide (PI) (Sigma) uptake as usual in our laboratory (Silva et al. 2010). This dye binds to double-stranded DNA and emits red fluorescence. At the end of the incubation periods, unpermeabilized adherent OPC cultured on coverslips were incubated with 75 μM PI fresh solution for 15 min at 37ºC in the absence of light. Cells were then fixed as described before and the nuclei stained with Hoechst 33258 dye. Red-fluorescence and U.V. images of fifteen random microscopic fields (original magnification: 400x) were acquired as described before. Number of total and PI+ cells was counted by investigators blinded as to the treatment received by cells and results expressed as fold vs. vehicle-treated cells. Loss of membrane integrity was further confirmed by scoring non-viable cells by trypan blue dye exclusion test and measuring lactate dehydrogenase (LDH) enzymatic activity in the incubation medium using a cytotoxicity detection kit and following manufacturer's instructions (Roche Molecular Biochemicals). All readings were corrected for the possible interference of UCB absorption and the amount of cell death (percentage) was calculated as released LDH/total LDH (value obtained by lysing all cells in the untreated wells with 2% Triton X-100 in DMEM for 5 min).

In addition, phycoerythrin-conjugated annexin V (V-PE) and 7-amino-actinomycin D (7-AAD) (Guava Nexin® Reagent, #4500-0450, Millipore) were used to

determine the percentage of viable, early-apoptotic and late-apoptotic/necrotic cells by flow cytometry. After incubation OPC were trypsinized, added to the cells present in the incubation media and then stained with annexin V-PE and 7-AAD, following manufacturer's instructions, and analysed on a Guava easyCyte 5HT flow cytometer (Guava Nexin® Software module, Millipore). Three populations of cells can be distinguished in this assay: viable cells (annexin V-PE and 7-AAD negative), early apoptotic cells (annexin V-PE positive and 7-AAD negative), and late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

2.5. Caspase activity determination

Activities of caspase-2, -3, and -9 were measured in total cell lysates by a colorimetric method as usual in our laboratory (Vaz et al. 2011b), according to the manufacturer's instructions. In brief, cells were harvested, washed with ice-cold PBS and lysed for 30 min on ice in the lysis buffer [50 mM 4-(2-hidroxyethyl)-1-piperazineethanosulfonic (HEPES) (pH 7.4), 100 mM NaCl, 0.1% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS), 1 mM dithiothreitol (DTT), 0.1 mM diaminoethanetetraacetic acid (EDTA)]. The proteolytic reaction was carried out in protease assay buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% (w/v) CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% (v/v) glicerol], containing 2 mM of specific substrate: Ac-VDVAD-pNA for caspase-2, Ac-DEVD-pNA for caspase-3 and Ac-LEHD-pNA for caspase-9 (Calbiochem, San Diego, CA, USA). Following incubation of the reaction mixtures for 2 h at 37 °C, the formation of pNA was measured in a microplate reader (PR 2100, BioRad Laboratories, Inc.) at λ=405 nm with a reference filter of 620 nm. Readings were normalized to total protein content determined using Bio-Rad's Protein Assay (Bio-Rad, Hercules, CA, USA) and expressed as fold change vs. vehicle-treated cells.

2.6. Assessment of mitochondrial dysfunction

After incubation with UCB, individual coverslips were rinsed with DMEM, loaded with 500 nM Mitotracker® Red CMXRos (in DMEM) (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C in the absence of light, and fixed as described before. Mitotracker® Red CMXRos is a cell-permeant dye, which passively diffuses across the plasma membrane and accumulates in active mitochondria. The intensity of the fluorescence emission was quantified per cell in at least two hundred cells with bipolar morphology per sample, using image analyser software (CSIRO HCA – Vision V 2.0.0).

For quantification of cellular red fluorescence from immunocytochemistry images, binary masks were created using the same cut-off intensity threshold value for each region of interest, which corresponds to cell cytoplasm, defined as the minimum intensity due to specific staining above background values. Results are expressed as fold vs. vehicle-treated cells.

2.7. Measurement of ROS production

The nonfluorescent dihydrorhodamine (DHR) 123 easily crosses cell membranes due to its lipophilicity and is converted by intracellular generated ROS into rhodamine 123, a fluorescent compound that accumulates in mitochondria and is considered as a sensitive indicator of ROS generation in cell systems (Gomes et al. 2005). To evaluate the production of ROS in OPC cultures, cells seeded on glass coverslips were loaded with 10 μM DHR 123 (Sigma) for 30 min at 37 °C prior to cellular treatment with UCB under light protection, as usual in our laboratory (Vaz et al. 2011a). At the end of the incubation periods, cells were fixed as previously indicated. Fluorescence intensity was quantified in at least two hundred cells, using the same software and specific binary masks, as described above for Mitotracker® fluorescence measurement. Since UCB was referred as an autofluorescent molecule in the presence of HSA (Ozkan et al. 1995), we performed in parallel a set of experiments in the absence of DHR 123. No variations in the fluorescence intensity were noticed in these experiments, which guarantee that the rise in the fluorescence intensity observed in the UCB-treated samples derives from ROS formation and not from UCB interference.

2.8. Measurment of calpain activation

After treatment with UCB, individual coverslips were rinsed with DMEM, labelled with the calpain substrate CMAC, t-BOC-Leu-Met (10 μM in DMEM) (Molecular Probes) for 30 min at 37 °C in the absence of light, and fixed as described before. Cellular blue fluorescence was visualised and the intensity of the fluorescence emission was quantified in at least two hundred cells, as before. Fragments of α-spectrin, obtained after calpain proteolysis (150 and 145 kDa fragments), were analysed in total cell lysates by Western blot. Results are expressed as fold vs. vehicle-treated cells.

2.9. Western blot assay

For Western blot analysis, cells were washed in ice-cold PBS, lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethylene glycoltetraacetic acid, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptine and 1 mM phenylmethylsulfonyl fluoride, and sonicated for 20 s. The lysate was centrifuged at 14,000 g for 10 min at 4ºC and the supernatants were collected and stored at -80ºC. Protein concentrations were determined as indicated before. Cell extracts containing equal amounts of protein (50 µg) were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk, incubated with the primary antibody overnight at 4ºC [mouse anti-KDEL (1:500) (#ab12223, AbCam), rabbit anti-IRE-1α (1:1,000) (#3294, Cell Signaling, Beverly, MA, USA), rabbit anti-ATF-6 (1:200) (#sc-166659, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-α-spectrin (1:500) (#MAB1622, Millipore), mouse anti-P-JNK1/2 (1:500) (#sc-81502, Santa Cruz Biotechnology) or mouse anti-β-actin (1:5,000) (Sigma)], and then with a horseradish peroxidase-labelled secondary antibody for 1 h at room temperature. After extensive washes, immunoreactive bands were detected by LumiGLO® (Cell Signaling) and visualized by autoradiography with Hyperfilm ECL. Results were normalized to β-actin expression and expressed as fold vs. vehicle-treated cells.

2.10. Densitometry and statistical analysis

The relative intensities of protein bands were analysed using the Quantity One (version 4.6) program (Bio-Rad).

Results of, at least, four different experiments were expressed as mean ± SEM. Significant differences between groups were determined by the two-tailed *t*-test performed on the basis of equal and unequal variance or by one-way ANOVA with Dunnett's or Bonferroni's multiple comparisons post-tests, using GraphPad PRISM 5.0 (GraphPad Software, San Diego, CA, USA), as appropriate and considering a minimal criterion of $P<0.05$ as statistically significant.

3. Results

3.1. Phenotypic characterization of enriched populations of OPC

At 6 DIV after isolation, enriched OPC culture was characterized using specific cell markers (Fig. II.1).

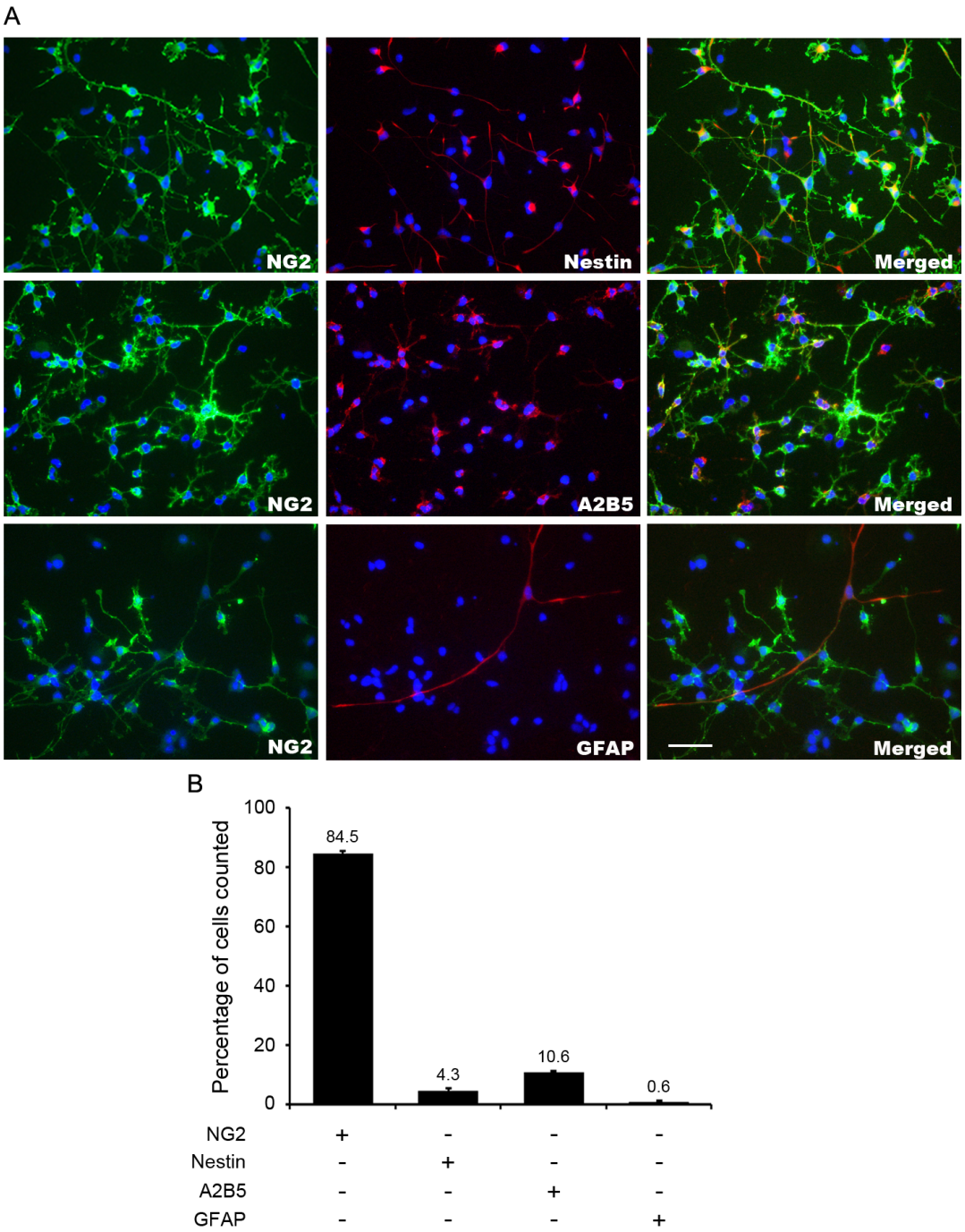


Fig. II.1 – Phenotypic characterization of the enriched culture of oligodendrocyte precursor cells obtained from neonatal rats. Nuclei were stained with Hoechst (blue) and cells labelled for NG2 (oligodendrocyte precursor cells), nestin (neural precursor cells), A2B5 (preoligodendrocytes) and glial fibrillary acidic protein (GFAP; astrocytes) as described in Materials and methods. **a** Representative results of one experiment are shown. Merged figures at right display the cells that simultaneously stain for NG2 and nestin (top), for NG2 and A2B5 (middle) or cells that stain only for NG2 and GFAP separately (bottom). **b** Graph bars represent the percentage of cells (mean ± SEM) positive for NG2, nestin, A2B5 or GFAP relatively to the total number of nuclei, from at least four independent experiments performed in duplicate. Scale bar represents 50 μm.

The majority of the cells (84.5%) presented bipolar morphology and stained for NG2 as depicted in Fig. II.1. A minor fraction was OL in a back stage positive for nestin

(4.3%) and preoligodendrocytes positive for A2B5 (10.6%). Moreover, we found that 45.7% of the cells simultaneously stained for NG2 and nestin (cells in a far premature phase), while 38.6% were positive for both NG2 and A2B5 (cells in a rather marginal advanced stage of differentiation than OPC), as displayed in Fig. II.1a (right, merged figures). Only a few number of cells (0.6%) were GFAP+ with typical astrocytic morphology and stained independently of OPC as expected (right, merged figure). No MBP+ or Iba-1+ cells were observed, indicating that both mature OL and microglia, respectively, were absent in our OPC culture Thus, our primary rat OPC culture yielded a very high number of OPC with scarce OL in other developmental stages.

3.2. UCB causes a decrease in OPC viability

First, we investigated whether incubation with UCB:HSA=0.5 for 2, 4, 6, 8 and 24 h increased OPC demise by apoptosis and necrosis-like cell death. DNA fragmentation, one hallmark of apoptosis, was examined by TUNEL assay. UCB increased TUNEL staining from 2 to 8 h of exposure, with peak values at 6 h (1.6-fold, $P<0.01$), as represented in Figs. II.2a, b. Activation of caspase-3, considered a central regulator of apoptosis, consistently increased from 2 h (1.6-fold, $P<0.01$) to 24 h (2.1-fold, $P<0.01$) of UCB exposure (Fig. II.2c), indicating that for later time points cells may be already committed to dye by apoptosis although the DNA fragmentation, measured by TUNEL assay, did not yet occurred.

Since apoptosis often coexists with necrosis-like cell death, we also used PI to selectively stain dead cells. As evidenced in Figs. II.3a, b, UCB significantly increased PI uptake from 4 to 8 h of exposure, with peak levels at 6 h (1.7-fold, $P<0.01$), corroborating the results of the TUNEL assay. The decrease in PI and TUNEL positive cells observed from 6 to 24 h of incubation may result from cells that have detached from the culture plate into the incubation medium, once both TUNEL and PI assays are for adherent cells. Thus, we next evaluated the number of OPC in the incubation medium by using the trypan blue exclusion test both in vehicle- (basal media containing HSA) or UCB-treated cells. The number of detached cells was similar in the presence or in the absence of UCB during the first 6 h of incubation (less than 10×10^3 OPC/ml). However, at 8 h and mainly at 24 h of incubation, increased number of cells was found in the incubation medium, with values significantly higher in the presence of UCB as compared with vehicle-treated cells (99.5×10^3 OPC/ml vs. 66.5×10^3 OPC/ml, respectively, $P<0.01$) (data not shown). To corroborate this finding we determined the release of LDH to cell supernatants and we have observed that UCB increases the

LDH release by more than 17% as compared to vehicle values (P<0.01) at 24 h (Fig. II.3c).

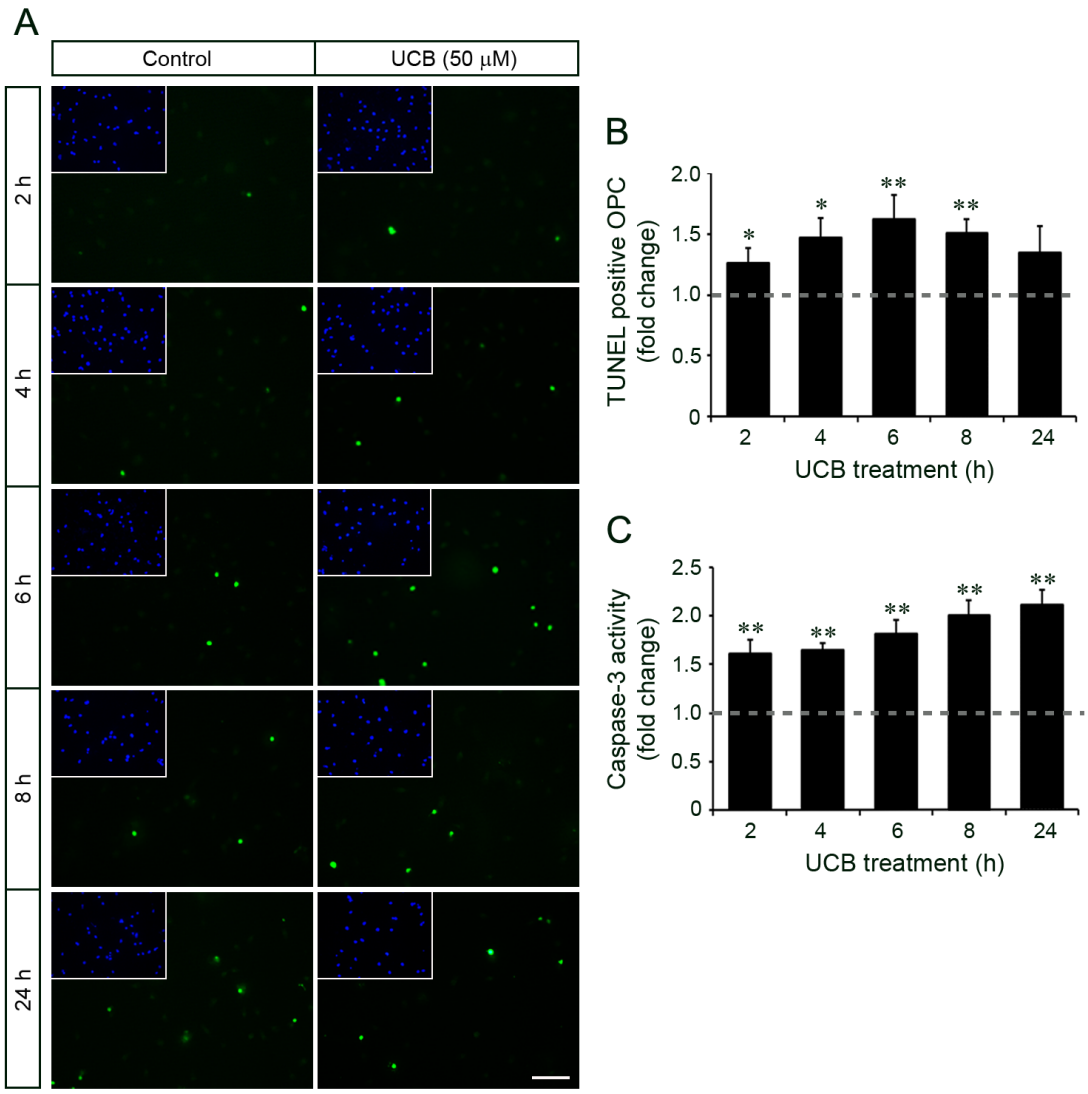


Fig. II.2 – Unconjugated bilirubin (UCB) induces cell death by apoptosis in oligodendrocyte precursor cells (OPC). Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of one experiment in cells treated with UCB and incubated with TUNEL (green) are shown. Insets evidence nuclei in the same fields stained with Hoechst (blue). **b** and **c** graph bars represent the fold increase from vehicle (mean ± SEM) for TUNEL assay and for caspase-3 activity, respectively, determined as indicated in Materials and methods, from at least four independent experiments performed in duplicate. Scale bar represents 50 μm. **P<0.01 and *P<0.05 vs. respective vehicle.

Given the differences obtained by apoptotic- vs. necrotic-related assays, we decided to evaluate the deleterious effects of UCB in OPC viability by flow cytometry following staining with annexin V-PE and 7-AAD, to differentiate the total amount of cells (adherent plus detached) into viable, early apoptotic and late apoptotic/necrotic cells. As shown in Table II.1, UCB decreased the number of viable cells from 2 to 24 h

of exposure (72.9% at 2 h to 67.3% at 24 h, *P*<0.01). Regarding cell death, UCB increased the percentage of OPC in early apoptosis until 6 h treatment (20.2%, *P*<0.01) corroborating TUNEL observations. In addition, UCB also promoted a time-dependent increase of OPC number in late apoptosis/necrosis stage (from 5.4% at 2 h to 11.8% at 24 h, *P*<0.01), which reflects the increase of caspase-3 and LDH release along the incubation period.

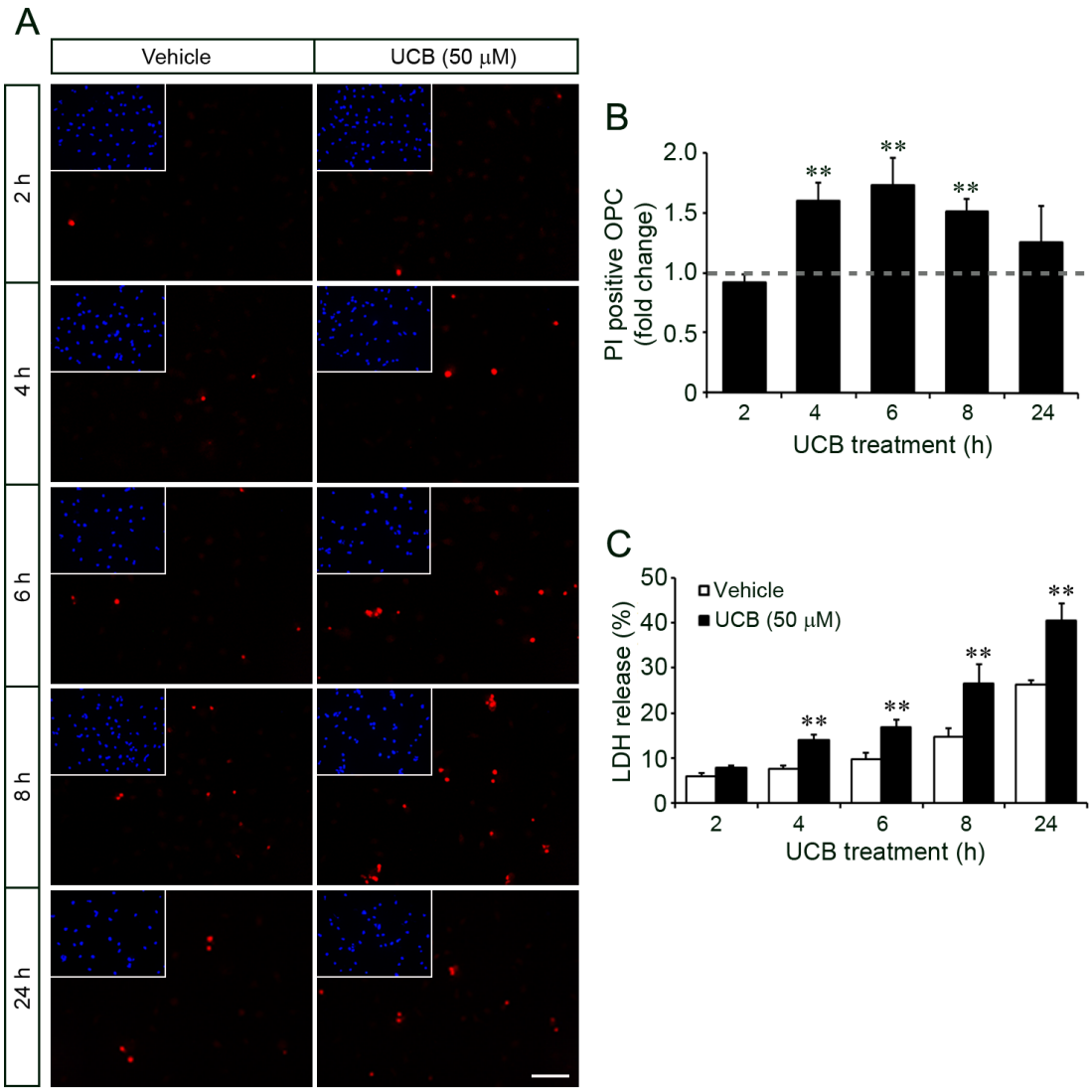


Fig. II.3 – Unconjugated bilirubin (UCB) induces necrosis-like oligodendrocyte precursor cell (OPC) death. Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of one experiment in cells treated with UCB and incubated with 75 μM propidium iodide (PI) (red) are shown. Insets evidence nuclei in the same fields stained with Hoechst (blue). **b** Graph bars represent the fold increase from vehicle (mean ± SEM) for PI+ cells, determined as indicated in Materials and methods, from at least four independent experiments performed in duplicate. **c** Graph bars represent the quantification of released lactate dehydrogenase (LDH) activity in the incubation medium. Scale bar represents 50 μm. ***P*<0.01 vs. respective vehicle.

Table II.1 – Temporal changes in oligodendrocyte viability by incubation with unconjugated bilirubin

	Viable		Early-apoptosis		Late-apoptosis/necrosis	
	Control	UCB	Control	UCB	Control	UCB
2 h	82.1 (±1.4)	72.8 (±2.7)*	9.1 (±1.0)	15.5 (±1.5)**	3.2 (±0.3)	5.4 (±0.8)*
4 h	81.5 (±2.1)	70.8 (±2.4)*	10.4 (±1.5)	19.4 (±2.5)*	3.5 (±0.5)	5.9 (±0.6)*
6 h	80.5 (±1.8)	70.0 (±2.5)**	13.4 (±0.8)	20.2 (±1.0)**	3.3 (±0.5)	8.0 (±0.7)**
8 h	80.5 (±3.2)	66.6 (±2.4)*	16.7 (±0.7)	21.6 (±3.8)	3.1 (±0.5)	8.4 (±0.7)**
24 h	77.1 (±1.5)	67.3 (±0.9)**	15.4 (±3.1)	16.8 (±1.4)	3.6 (±0.8)	11.8 (±0.6)**

All results are mean ± SEM from at least five independent experiments. Oligodendrocyte precursor cells were incubated without (control) or with 50 μM unconjugated bilirubin (UCB), in the presence of 100 μM human serum albumin. The percentage of viable oligodendrocytes and oligodendrocytes in early- or late-apoptosis/necrosis were determined by flow cytometer with phycoerythrin (PE)-conjugated annexin V and 7-amino-actinomycin D (7-AAD). ***P*<0.01 and **P*<0.05 vs. respective vehicle.

In addition, to attest the involvement of caspase-3 activity in UCB-induced cell dead, OPC were treated with Z-VAD-FMK (1 μM), a cell-permeable pan-caspase inhibitor, during UCB treatment that resulted in the prevention of UCB-induced caspase-3 activation (Suppl. Fig. II.1). Then cells were analysed using the flow cytometer technique described above at 6 h (peak values for OPC in early apoptosis) and 24 h (peak values for cells in late apoptosis/necrosis). As shown in Table II.2, Z-VAD-FMK reduced the percentage of cells in early (minus 9.5% and 6.1% of cells at 6 h and 24 h, respectively, $P<0.01$) and late-apoptosis/necrosis (minus 1.5% and 4.9% of cells at 6 h and 24 h, respectively, $P<0.05$).

Table II.2 – Oligodendrocyte precursor cell death by unconjugated bilirubin is partially prevented by Z-VAD-FMK, N-acetylcysteine (NAC) and calpeptin

		Viable		Early-apoptosis		Late apoptosis/necrosis	
		Control	UCB	Control	UCB	Control	UCB
6 h	Pre-test	80.5 (±1.8)	70.0 (±2.5)**	13.4 (±0.8)	20.2 (±1.0)**	3.3 (±0.5)	8.0 (±0.7)**
	Z-VAD-FMK	80 (±2.1)	75.1 (±1.9)	12.6 (±1.5)	10.6 (±1.1)##	2.6 (±0.5)	6.5 (±0.7)
	NAC	85.3 (±1.0)	77.3 (±0.5)	13.7 (±2.2)	9.4 (±3.2)##	2.1 (±0.3)	6.1 (±0.5)
	Calpeptin	80.7 (±2.1)	80.9 (±1.1)#	11.7 (±0.2)	13.1 (±0.7)##	3.2 (±0.2)	5.5 (±1.0)
24 h	Pre-test	77.1 (±1.5)	67.3 (±0.9)**	15.4 (±3.1)	16.8 (±1.4)	3.6 (±0.8)	11.8 (±0.6)**
	Z-VAD-FMK	84.4 (±0.9)**	78.6 (±1.4)##	9.5 (±0.9)	10.8 (±0.8)##	3.3 (±0.2)	6.1 (±0.5)##
	NAC	80.8 (±1.3)	78.7 (±1.2)##	12.0 (±0.6)	11.7 (±1.0)#	4.2 (±0.5)	6.2 (±0.8)##
	Calpeptin	80.9 (±0.9)	77.3 (±1.9)##	11.8 (±0.6)	12.1 (±0.7)#	3.8 (±0.3)	6.1 (±0.8)##

All results are means ± SEM from at least four independent experiments performed in duplicate. Oligodendrocyte precursor cells from rat cortex were incubated without (control) or with 50 μM unconjugated bilirubin (UCB), in the presence of 100 μM human serum albumin for 6 and 24 h. In parallel experiments cells were incubated with 1 μM Z-VAD-FMK (caspase inhibitor), 100 μM N-acetylcysteine (NAC) (antioxidant) and 5 μM calpeptin (calpain inhibitor). The percentage of viable oligodendrocytes and oligodendrocytes in early- or late-apoptosis/necrosis were determined by flow cytometer with phycoerythrin (PE)-conjugated annexin V and 7-amino-actinomycin D (7-AAD).
***P*<0.01 vs. respective control; ##*P*<0.01 and #*P*<0.05 vs. respective Pre-test.

Taken together, these results demonstrate that UCB reduces the OPC viability by both apoptosis and necrosis-like cell death, which is directly related with the time of incubation.

3.3. UCB increases the production of ROS by OPC, but not that of cytokines or glutamate

As we observed a loss in OPC viability by exposure to UCB, we decided to evaluate the intracellular pathways and subcellular compartments that could be implicated in such event. Thus, we assessed the ROS production, as well as the TNF-α, IL-1β and glutamate release, all of them recognized by their toxic properties to OPC (McTigue and Tripathi 2008). ROS production by treatment of OPC with UCB increased from 6 to 24 h (Fig. II.4) (>1.2-fold, $P<0.05$), indicating ROS as a key player in the mechanisms of OPC demise by both apoptotic and necrotic pathways.

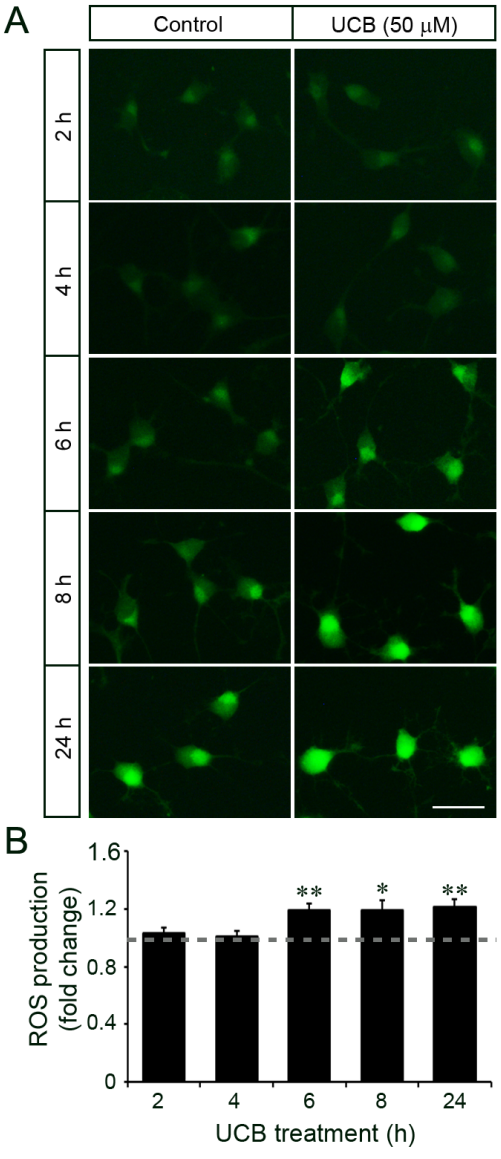


Fig. II.4 – Generation of reactive oxygen species (ROS) by oligodendrocyte precursor cells (OPC) is enhanced upon unconjugated bilirubin (UCB) stimulation. Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of one experiment in cells treated with UCB and incubated with 10 μM dihydrorhodamide 123 (green), as described in Material and methods, are shown. **b** Graph bars represent the fold increase from vehicle (mean ± SEM) in ROS content evaluated by fluorescence intensity from at least four independent experiments performed in duplicate. Scale bar represents 20 μm. $^{**}P<0.01$ and $^{*}P<0.05$ vs. respective vehicle.

In an effort to establish a link between oxidative stress and UCB toxicity, cultures were treated with 100 μM NAC for 24 h with or without UCB. NAC, a precursor of the antioxidant glutathione, efficiently prevented UCB-induced ROS generation (Suppl. Fig. II.1). As stated in Table II.2, incubation with NAC significantly reduced the amount of OPC in early apoptosis (minus 7.7% and 4.8% at 6 h and 24 h, respectively, $P<0.01$). Interestingly, NAC only reduced late-apoptosis/necrosis for the extended incubation period (minus 4.9% at 24 h, $P<0.01$), thus indicating an association with the marked production of ROS observed at the same stage.

In contrast, release of TNF-α, IL-1β and glutamate remained unchanged following OPC incubation with UCB (data not shown). These findings reveal that OPC behave differently from astrocytes and microglia upon UCB stimulus, namely regarding the secretion of pro-inflammatory cytokines and glutamate (Brites 2011).

3.4. UCB triggers mitochondrial dysfunction and caspase-9 cascade activation in OPC

During cellular stress mitochondrial membrane suffers depolarization resulting in the release of ROS and caspase-dependent or -independent apoptotic pathways (Sánchez-Gómez et al. 2003). Given our results indicating an increase in ROS generation, after OPC treatment with UCB, we next determined if mitochondrial activity in OPC was compromised by UCB. Therefore, we used the mitochondrial membrane potential-sensitive dye Mitotracker® Red CMXRos. As shown in Figs. II.5a, b, mitochondrial viability revealed to be reduced at 2 and 4 h after UCB stimulation (<0.9-fold, $P<0.01$). Although this effect appears to occur in a short period of time, it deserved to be noted that Mitotracker® Red CMXRos fluorescence in UCB-treated cells was below vehicle values until 6 h. In addition, from 8 h onwards also vehicle-treated cells showed a decrease in fluorescence intensity, which may account for the lack of differences between vehicle- and UCB-treated cells. Because caspase-9 is an apoptotic initiator protease activated subsequently to the mitochondrial damage we though to determine whether UCB was able to stimulate this protease. As expected, a marked increase in caspase-9 activity occurred at the same time points (Fig. II.5c) of the previous experiment, e.g. at 2 and 4 h OPC exposure to UCB (>1.5-fold, $P<0.01$). Our data thus demonstrate that the loss of mitochondria viability, with the consequent activation of caspase-9 cascade, may be another intervenient in the OPC death cascade by UCB. Indeed, the use of the pan-caspase inhibitor, which also prevented caspase-9 activation (Suppl. Fig. II.1), significantly inhibited UCB-induced OPC death (Table II.2), supporting the involvement of the mitochondria pathway in OPC demise.

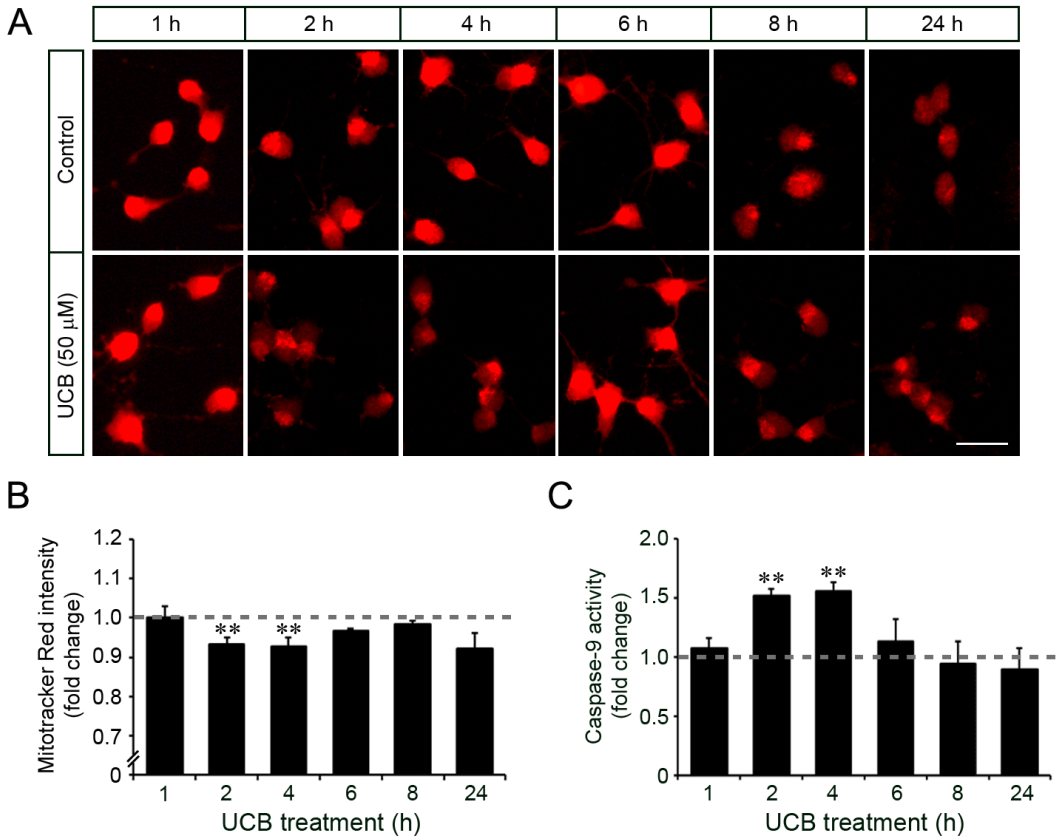


Fig. II.5 – Unconjugated bilirubin (UCB) decreases mitochondrial viability and activates caspase cascade in oligodendrocyte precursor cells (OPC). Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of one experiment in cells treated with UCB and incubated with 500 nM Mitotracker® Red CMXRos are shown. **b** and **c** Graph bars represent the fold increase from vehicle (mean ± SEM) in labelled active mitochondria evaluated by fluorescence intensity and in caspase-9 activity, respectively, determined as indicated in Materials and methods, from at least five independent experiments performed in duplicate. Scale bar represents 20 μm. ***P*<0.01 vs. respective vehicle.

3.5. UCB activates ER stress pathway in OPC

Ca^{2+} transfer from ER to mitochondria at contact sites between the organelles can induce mitochondrial dysfunction and programmed cell death after stress. Accordingly, in addition to displaying independent effects on apoptosis, the ER and mitochondria also exert a crosstalk during the execution of the apoptosis (Verma and Datta 2011). Because injury-related unfolded protein response triggers the activation of multiple ER-resident sensors, including IRE-1α and ATF-6 and the consequent release of GRP78, we determined if UCB would cause ER stress response and upregulation of these chaperones using specific antibodies. The first protein to be overexpressed was GRP78 at 1 h of UCB exposure (1.1-fold, *P*<0.01) (Figs. II.6a, b), immediately followed by a similar and more evident induced expression of both proteins, GRP78 and GRP94, at 2 h of OPC treatment with UCB (1.3-fold and 1.4-fold, respectively,

P<0.01). Up-regulation of these ER chaperones (1.5-fold, *P*<0.05) were also observed at 8 h (GRP78) and at 24 h (GRP94), probably as a secondary effect of ROS generation triggered by UCB (Fig. II.4). Since changes in GRP78 and GRP94 expression were modest, we decided to evaluate also the expression of ATF-6 and IRE-1α (Suppl. Fig. II.2). UCB treatment was able to induce a slight increase at 1 h in ATF-6 (1.1-fold, *P*<0.05) and IRE-1α (1.2-fold, *P*<0.01), followed by a more evident induced expression of IRE-1α at 2 h (1.3-fold, *P*<0.01). A second upregulation of IRE-1α was also observed at 24 h (1.2-fold, *P*<0.05). Nevertheless, ATF-6 and IRE-1α expression was shown to vary along the time course of the experiment even in vehicle-treated cells indicating a more transient expression of these chaperones along the incubation period. Our data suggest that UCB is able to promote a faster expression of IRE-1α, while for ATF-6 UCB-induced expression occurred at the same time but with higher levels. As depicted in Fig. II.4a and Suppl. Fig. II.2a the levels of all these chaperones also suffered a variation in vehicle-treated cells. This could be ascribed to the effect of HSA on OPC, since this molecule is used in the incubation medium to avoid UCB precipitation along the time of incubation.

The initiator caspase-2 was demonstrated to be involved in OL apoptosis (Gu et al. 1999). This ER-resident premitochondrial protease mediates apoptosis through ER stress-dependent signalling (Upton et al. 2008). Therefore, we next determined UCB ability to activate caspase-2 in OPC and found that UCB causes an early activation of caspase-2 starting at 1 h and increasing in a time-dependent fashion up to 8 h (3.8-fold, *P*<0.01), although maintained activated until 24 h incubation (Fig. II.6c). The profile obtained for the activation of caspase-2 by UCB in OPC suggests that other intracellular mechanisms rather than ER stress will be involved, including stress in other organelles or UCB-induced ROS consequences.

Globally, these findings suggest that the early stage of OPC toxic stress by UCB is caused at the level of the ER, determining a subsequent unfolded protein response, which may play a pivotal role in the pathology of BIND, at least in what relates to myelinogenesis.

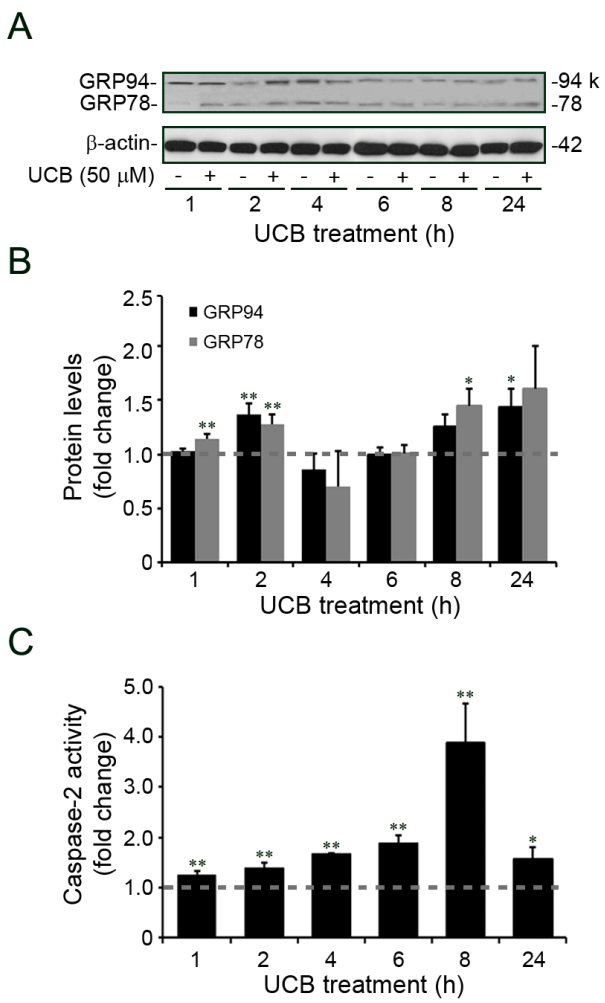


Fig. II.6 – Unconjugated bilirubin (UCB) induces endoplasmic reticulum stress in oligodendrocyte precursor cells (OPC). Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of glucose-regulated protein (GRP) 78 and GRP94 expression by Western blot using specific antibodies are shown. **b** Graph bars represent the fold increase from vehicle (mean ± SEM) obtained for protein band intensity by scanning densitometry standardized with respect to β-actin protein for GRP94 and GRP78 determined as indicated in Materials and methods, from at least four independent experiments. **c** Graph bars represent the fold increase from vehicle (mean ± SEM) for caspase-2 activation using a colorimetric substrate cleavage assay, from at least four independent experiments performed in duplicate. ***P*<0.01 and **P*<0.05 vs. respective vehicle.

3.6. UCB triggers the activation of JNK signalling pathway in OPC

Previous reports suggested that ER stress triggers caspase-2 activity through the JNK activation (Murakami et al. 2007) leading to apoptosis, while others indicate JNK as one of the downstream targets of caspase-2 (Panaretakis et al. 2005). We have demonstrated that JNK1/2 signalling pathways play a key role in mediating the reactivity of astrocytes and microglia to UCB (Fernandes and Brites 2009; Silva et al. 2010). Nevertheless, JNK1/2 involvement in OPC survival following UCB exposure was never investigated. So, we assessed the phosphorylated (activated) form of this kinase in total cell lysates of UCB-exposed OPC by Western blot, using a specific antibody. As depicted in Fig. II.7, JNK 1/2 is highly activated at 1 h in both vehicle- and UCB-treated cells; to this feature it may account the basal JNK activity observed in OPC (Pirianov et al. 2006) and the effect of the PDGF on this kinase (Chew et al. 2010). Regarding to UCB effect, once again and similarly to the upregulation of GRP78 and GRP94, two time-frames of UCB-induced activation of JNK1/2 were noticed (Fig. II.7), one more

robust at 2 h incubation (1.4-fold, *P*<0.01) and another at 8 h of UCB treatment (1.2-fold, *P*<0.01).

The similarity of the time-frames between GRP78 upregulation and JNK activation and the fact that both effects occur in parallel to mitochondrial dysfunction and increased caspase-9 activity suggest a functional association. Therefore, it seems that the activation of both ER stress and JNK pathways may also amplify the proapoptotic signals by interacting with mitochondria and eliciting the activation of another initiator caspase.

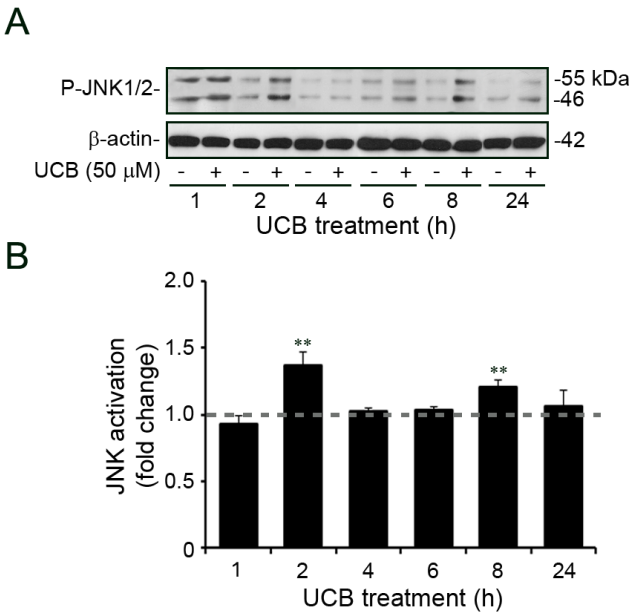


Fig. II.7 – Unconjugated bilirubin (UCB) stimulates the activation of JNK pathway in oligodendrocyte precursor cells (OPC). Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of the phosphorylated form of P-c-Jun *N*-terminal kinase (JNK) 1/2 in total cell lysates analysed by Western blot using a specific antibody are shown. **b** Graph bars represent the fold increase from vehicle (mean ± SEM) obtained for protein band intensity by scanning densitometry standardized with respect to β-actin from at least four independent experiments performed in duplicate. ***P*<0.01 vs. respective vehicle.

3.7. UCB upregulates calpain activity in OPC

Finally, because mitochondria and ER are co-involved in the homeostasis of Ca^{2+}, we decided to investigate the role of calpain, a Ca^{2+}-activated neutral proteinase in OPC death by UCB. By using a specific substrate, which acquires fluorescence upon calpain proteolytic cleavage, we observed an increase in calpain activity at 4, 6 and 24 h exposure of OPC to UCB (Figs. II.8a, b), with maximal levels at 4 h (1.3-fold, *P*<0.01).

To reassure that UCB triggers calpain activation in OPC, we also evaluated the cleavage of α-spectrin, its preferred substrate, by Western blot. Immunoblotting showed that α-spectrin cleavage occurred through the generation of 150 KDa (proteolyzed by caspase-3 and calpain) and 145 KDa (calpain specific) breakdown products as previously reported (Chen et al. 2011) for both vehicle- and UCB-treated

cells, suggesting once again that OPC may also be responding to the HSA content in vehicle. This cleavage can occur as a physiological response, since calpains are a family of Ca^{2+}-dependent proteases that are ubiquitously and constitutively expressed in mammalian cells. They cleave a number of cellular substrates, including kinases, phosphatases, transcription factors, and cytoskeletal proteins. In this context, some studies have demonstrated that minor activation of these proteases triggers cell migration, membrane protrusion and proliferation (Franco et al. 2004; Goll et al. 2003; Carragher and Frame 2002; Sorimachi et al. 1997). Nevertheless, UCB induction over vehicle values was observed at 6 h incubation (1.5-fold, $P<0.01$) (Fig. II.8c). The obtained profile is similar to that of calpain activation, although more elevated and slightly delayed. Even if we only analyse the calpain-specific 145 kDa fragment, a comparable pattern is still obtained (data not shown).

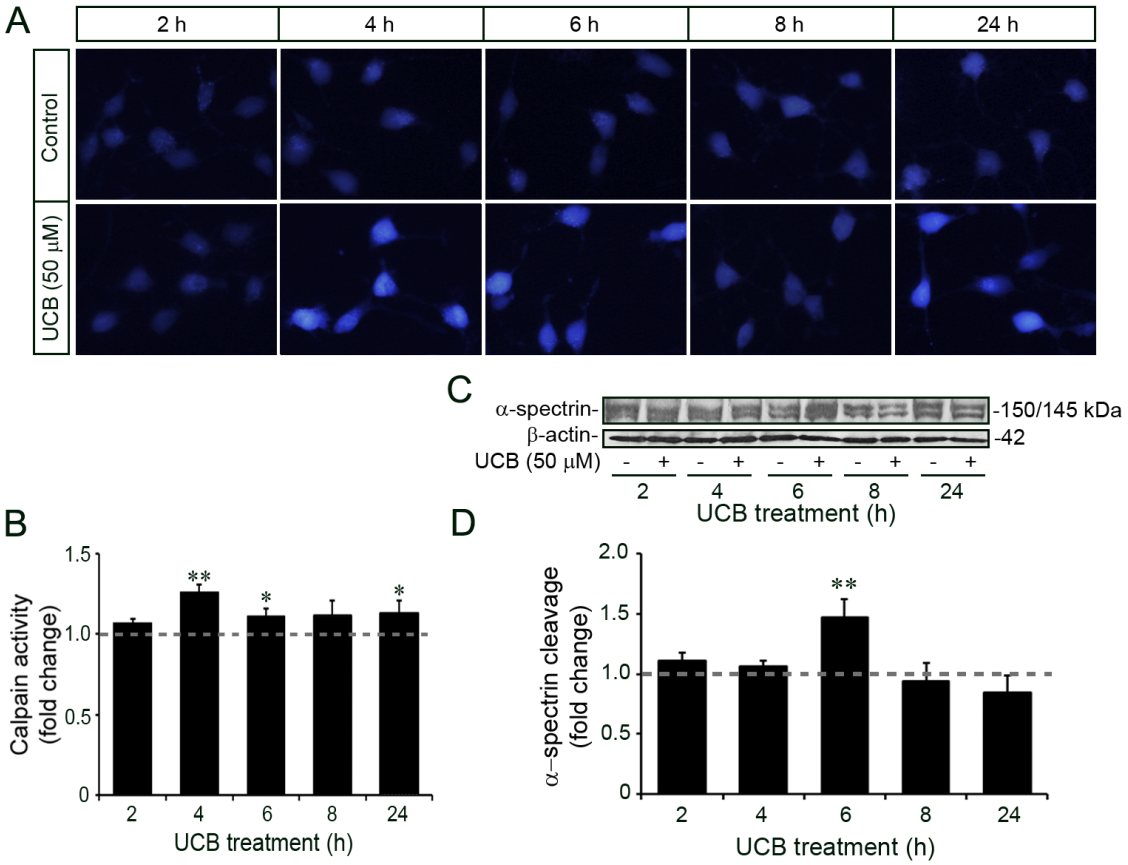


Fig. II.8 – Calpain activation in oligodendrocyte precursor cells (OPC) increases upon exposure to unconjugated bilirubin (UCB). Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of one experiment in cells treated with UCB and incubated with 10 μM specific calpain substrate CMAC, t-BOC-Leu-Met, are shown. **b** Graph bars represent the fold increase from vehicle (mean ± SEM) for calpain activation from at least three independent experiments performed in duplicate. **c** Representative results of the cleavage products 150 kDa and 145 kDa from α-spectrin in total cell lysates analysed by Western blot using anti-α-spectrin antibody are shown. **d** Graph bars represent the fold increase in intensity from vehicle (mean ± SEM) obtained for α-spectrin cleaved peptide bands using scanning densitometry standardized to β-actin, from at least four independent experiments performed in duplicate. Scale bar represents 20 μm. ***P*<0.01 and **P*<0.05 *vs.* respective vehicle.

To further reinforce the role of calpains in UCB-induced OPC death, we co-incubated parallel cultures with 5 μM calpeptin, a calpain inhibitor that revealed efficacy in inhibiting UCB-induced calpain activation (Suppl. Fig. II.1). As shown in Table II.2, calpeptin reduced the percentage of cells in early-apoptosis (minus 7.1% and 4.7% of cells at 6 h and 24 h, respectively, $P<0.01$) and late-apoptosis/necrosis (minus 2.5% and 5.7% of cells at 6 h and 24 h, respectively, $P<0.05$).

Therefore, we may assume that inhibitory effects exerted by caspase and calpain inhibitors, as well as by NAC indicate that activation of caspases and calpains, together with oxidative stress are involved in UCB-induced OPC apoptosis and necrosis-like cell demise. Taken together, the results indicate that OPC death by UCB is mediated by different pathways, and that a combination of several protective strategies would be valuable to prevent the multiple types of damage that may occur *in vivo* during neonatal hyperbilirubinemia and associated impairment of myelin formation.

4. Discussion

Although UCB was indicated as causing impairments in myelination (Ahdab-Barmada and Moossy 1984; Brito et al. 2011) it was never investigated whether UCB impairs the proliferation of OPC that generate myelin-forming OL. Our data demonstrate that exposure of primary OPC to UCB in conditions mimicking a moderate neonatal hyperbilirubinemia (UCB/HSA=0.5) decreases OPC survival, which may impair myelination. Indeed, myelination mostly occurs in early postnatal days (Limperopoulos 2010), involving OPC differentiation into immature OL during the suckling period and then into myelinating mature OL. Thus, although developmental defects of myelin are not lethal in prenatal life, they are considered a cause of neurodevelopmental diseases presenting an altered myelination phenotype (Nave 2010), given that OL will also fail in supporting the long-term integrity of axons (Kassmann and Nave 2008). Therefore, pathological alterations that disturb life/death decisions in the OL lineage may lead to a reduction in the number of myelinating cells relatively to that of axons, resulting in dys- and demyelinating disorders. Between the most considered cell death initiators of the OL lineage are progressive mitochondrial dysfunction and ER stress (Casaccia-Bonnefil 2000). Here, we demonstrate that UCB triggers apoptotic and necrotic-like OPC demise mediated by ER stress, mitochondrial dysfunction, JNK phosphorylation and calpain activation.

We and others have been studying the toxic effects of UCB in neurons, astrocytes and microglia (Deganuto et al. 2010; Brites 2011), while only one study have

previously demonstrated that UCB decreases mature OL viability in a concentration and time-dependent manner (Genc et al. 2003). OPC are the most abundant cells of the OL lineage during the perinatal period, and accordingly that should be considered a potential target to UCB effects during neonatal hyperbilirrubinemia. In addition, OPC are more vulnerable than mature OL to several stimuli such as oxygen-glucose deprivation (Deng et al. 2003) and pro-inflammatory cytokines (Deng et al. 2008) what turns OPC particularly suitable to evaluate potential pathological effects determined by common conditions in neonatal life.

Although the apoptotic features were observed earlier than the necrosis-like cell death, our results clearly showed that UCB-induced apoptotic and necrotic-like OPC features are time-dependent and corroborate similar findings in astrocytes, microglia, neurons and brain endothelial cells (Falcão et al. 2006; Silva et al. 2010; Palmela et al. 2011). First appearance of apoptosis occurs by the externalization of phosphatidylserine, usually evaluated by annexin V binding, which we observed after the incubation of synaptosomes with UCB (Brito et al. 2004) and with immature neurons for short exposure periods (Vaz et al. 2010). In addition, caspase-3 activation along the incubation period, one effector caspase of the apoptotic cell death cascade, may justify the observed time-dependent increase of late-apoptotic/necrotic OPC. A prompt activation of this caspase was also observed in microglia, neurons and brain endothelial cells exposed to UCB (Silva et al. 2010; Palmela et al. 2011; Vaz et al. 2010), pointing to caspase-3 activation as a characteristic feature of UCB-induced activation of the apoptotic machinery. The use of the pan-caspase inhibitor further confirmed caspase-3 involvement in UCB-induced OPC demise. As observed in other nerve cells (Fernandes and Brites 2009), time of exposure is a crucial factor of OPC cell death, thus corroborating its role as a risk factor for BIND and a determinant for clinical intervention in order to prevent detrimental outcomes in severely ill neonates.

Formation of ROS has been indicated to mediate cytotoxicity to OL in many neurological disorders, and OPC are particularly susceptible to oxidative stress-mediated injury (McTigue and Tripathi 2008). Although not primarily found in astrocytes, ROS generation by UCB was previously noticed in both mature and immature neurons (Brites 2011; Vaz et al. 2011a). In accordance, our results showed that UCB induces ROS generation in OPC and incubation with NAC prevented OPC death supporting the involvement of ROS. Moreover, ROS production was particularly evident from 6 to 24 h incubation, suggesting that besides being a precipitating insult it is also a source of secondary OPC damage. Indeed, increased ROS levels are a cause and consequence of mitochondria dysfunction making them uniquely vulnerable to oxidative stress and damage (Waldbaum and Patel 2010), as it is also observed in OL

(Galluzzi et al. 2009). Therefore, mitochondrial dysfunction and oxidative stress by UCB mediate OPC death. This is not without precedence since it was demonstrated that cannabidiol induces OL cytotoxicity via mitochondrial dysfunction and ROS production (Mato et al. 2010) and our reports in the last years really demonstrated that mitochondrial dysfunction and oxidative stress are key players in neuronal injury by UCB, at least in *in vitro* models (Brites 2011; Vaz et al. 2011b).

OL, given their high metabolic rate, are particularly susceptible to disruptions of the secretory pathway and ER stress. This pathway, initiated by the accumulation of unfolded or misfolded proteins, leads to GRP78 release and consequent upregulation of the molecular chaperones ATF-6 and IRE-1α (Bernales et al. 2006; Romisch 2005; Ruiz et al. 2010). If this adaptive response is insufficient, the unfolded protein response activates an apoptotic program to eliminate damaged cells (Lin and Popko 2009). Only one recent study has demonstrated that ER stress triggers the upregulation of GRP78 in injured OPC by hypoxia and ischemia (Kraskiewicz and FitzGerald 2011). Our results showed that UCB activates the unfolded protein response in OPC by upregulating ATF-6, IRE-1α, GRP78 and GRP94. Interestingly, either UCB or free bilirubin (unconjugated and not bound albumin) upregulated many genes involved in ER stress both in SH-SY5Y (Calligaris et al. 2009) and Hepa 1c1c7 cells (Oakes and Bend 2010). In addition to unfolded protein response, results here reported suggest that UCB exposure also causes caspase-2 cleavage, which may be related to ER stress-induced apoptotic cell death. Indeed, caspase-2 activation can serve as a proximal caspase that function upstream of mitochondrial signalling during ER stress-induced apoptosis (Gu et al. 2008). ER stress-induced caspase-2 activation is required for mitochondrial transmembrane loss, cytochrome *c* release and its downstream caspase-9 activation in myeloma cells (Gu et al. 2008), as well as BID cleavage in mouse embryonic fibroblasts (Upton et al. 2008). Here we demonstrated that caspase-2 activation occurs from 1 h onwards with maximal levels at 8 h after UCB treatment suggesting a late and concomitant involvement of ER stress and JNK activation. Moreover, caspase-2 indicated as being located in the mitochondria and in the Golgi complex (Mancini et al. 2000; Murakami et al. 2007) may be activated by the subsequent ROS generation further contributing to its increase at 8 h of UCB exposure, as well as to the release of pro-apoptotic signals. In contrast, caspase-9 activation that was transiently observed at 2 and 4 h of OPC incubation with UCB, suggest a direct effect of UCB in the mitochondria as we demonstrated to happen at the complex IV of mitochondrial respiratory chain in immature neurons (Vaz et al. 2010). In fact, mitochondrial alterations are for long indicated as the earliest manifestations of bilirubin encephalopathy (Jew and Williams 1977). In addition, present results evidence that

UCB directly affects ER before acting on the mitochondria, since upregulation of GRP78, IRE-1α and ATF-6, as well as of caspase-2 activation were observed prior to mitochondrial dysfunction and caspase-9 activation.

Activation of JNK pathway was shown to depend on the cell type and stimuli. In PC12 cells, ER stress triggers caspase-2 activity through the JNK activation (Murakami et al. 2007), while in Jurkat WT and AS2 cells it does not occur upstream of caspase-2 (Panaretakis et al. 2005). Furthermore, the release of Ca^{2+} from ER may mediate the activation of JNK directly, triggering further release of Ca^{2+}, and JNK can critically mediate the subsequent ER-mitochondrial crosstalk (Verma and Datta 2011). Our study showed that JNK activation occurs after GRP78 upregulation and caspase-2 activation suggesting that it may be a downstream event of the ER stress. Nevertheless, we can also hypothesize that the rise in cytosolic Ca^{2+} from both ER and JNK activation once taken up by mitochondria originate the onset of apoptosis and a second cycle of ER stress. Hence, activation of JNK 1/2 by UCB may be associated to mitochondrial dysfunction and apoptosis of OPC, similarly observed in astrocytes, neurons and microglia (Fernandes and Brites 2009; Silva et al. 2010; Vaz et al. 2011b).

The sustained high concentration of intracellular Ca^{2+}, followed by activation of calpains, with neuronal death and axonal degeneration was observed in traumatic brain injury (Saatman et al. 2010), tauopathys (Liu et al. 2011) and Alzheimer's disease (Lopes et al. 2010), and calpain inhibitors evidenced to protect OL from cell death (Benjamins et al. 2003; Mato et al. 2010). As expected, we observed an increase in calpain activation at 4 h of OPC incubation with UCB while inhibition by calpeptin was able to prevent the OPC demise. Exposure of OL to diverse compounds revealed that calpains mediate the mitochondria-dependent death pathway (Sánchez-Gómez et al. 2011; Fu et al. 2009). Interestingly, the UCB-induced activation of calpains in hippocampal slice cultures mediates associated to the impairment of long-term synaptic plasticity (Chang et al. 2009). Therefore, activation of calpains by UCB may not only promote nerve cell death but also compromise cellular specific functions.

Taken together, UCB interaction with OPC causes a rapid increase in features of ER stress, consequent activation of JNK and mitochondrial dysfunction, and calpain activation and generation of ROS. This cascade of events culminates in OPC death by both apoptosis and necrosis, as schematically indicated in Fig. II.9. Interestingly, the marked prevention of UCB-induced OPC demise by all the inhibitors corroborate that caspase, ROS and calpain cascades evidence a cross-talk with one cascade acting laterally on another cascade. Once damage of UCB to OPC may alter the neurodevelopmental program and induce long-lasting myelination deficit, we believe that the present results will be of value to better understand the mechanisms leading to

BIND and impact on neurological handicaps in terms of susceptibility to ageing and adult brain diseases.

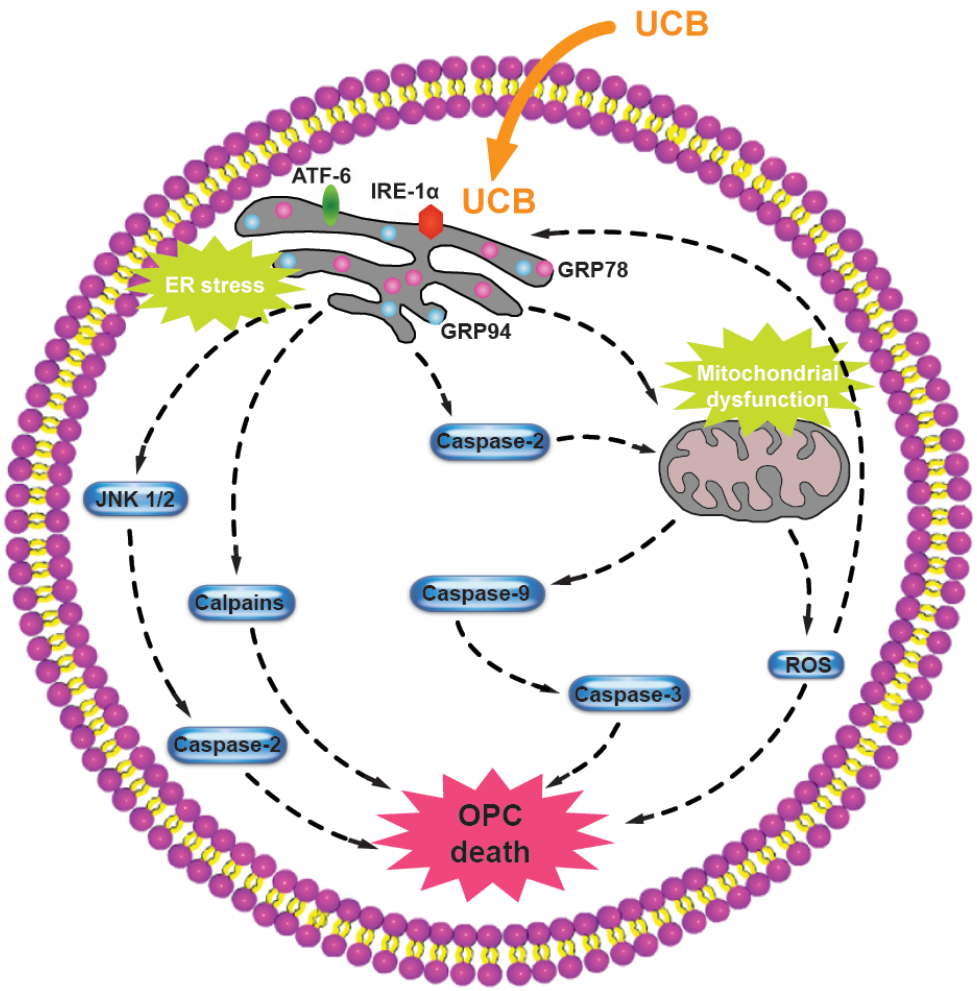


Fig. II.9 – Schematic representation of the potential intracellular pathways and subcellular compartments involved in unconjugated bilirubin (UCB) injury to oligodendrocyte precursor cells (OPC). UCB interaction with OPC causes a rapid increase of endoplasmic reticulum (ER) stress, with upregulation of chaperones inositol-requiring enzyme 1α (IRE-1α), activating transcription factor 6 (ATF-6), glucose-regulated protrein (GRP) 78 and GRP94 and caspase-2 activation), followed by mitochondrial dysfunction and consequent activation of caspase-9 and c-Jun *N*-terminal kinase (JNK) 1/2. Thus, UCB seems to directly interact with ER before acting on the mitochondria. Caspase-2 continuous activation may additionally derive from its different intracellular location, as a result of diverse stressed intracellular organelles and from JNK-signalling pathway, as a downstream target. Reactive oxygen species (ROS) generated by damaged mitochondria and from secondary injuries will concur for the second level of caspase-2 and JNK activation, as well as of unfolded protein upregulation, with further production of ROS as a byproduct of protein oxidation in ER. These mechanisms support the notion that OPC death by UCB is mediated by different pathways, involve early and late events as well as the dysfunction of several organelles, and that caspases and calpains are involved in the process of apoptosis and necrosis-like cell demise.

The overall findings appear compatible with the decreased density of myelinated fibers observed in the cerebellum of an infant with *kernicterus* (Brito et al. 2011), the separation of the myelin lamellae in a model of experimental *kernicterus*

(Chen et al. 1971) and the myelin figures surrounding vacuoles, bits of cytoplasm and other intracytoplasmic debris in the Gunn rat (Jew and Williams 1977). Further elucidation of the role of UCB on OPC differentiation into mature OL and their inability to properly myelinate, either in *in vitro* and most importantly in animal models of hyperbilirubinemia, may provide new evidences for a closer clinical surveillance intended to prevent detrimental outcomes produced by hyperbilirubinemia in the first week of postnatal life.

5. References

Ahdab-Barmada, M., & Moossy, J. (1984). The neuropathology of kernicterus in the premature neonate: diagnostic problems. J Neuropathol Exp Neurol, 43(1), 45-56.

Benarroch, E. E. (2009). Oligodendrocytes: Susceptibility to injury and involvement in neurologic disease. Neurology, 72(20), 1779-1785.

Benjamins, J. A., Nedelkoska, L., & George, E. B. (2003). Protection of mature oligodendrocytes by inhibitors of caspases and calpains. Neurochem Res, 28(1), 143-152.

Bernales, S., Papa, F. R., & Walter, P. (2006). Intracellular signaling by the unfolded protein response. Annu Rev Cell Dev Biol, 22, 487-508.

Bogler, O., Wren, D., Barnett, S. C., Land, H., & Noble, M. (1990). Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proc Natl Acad Sci U S A, 87(16), 6368-6372.

Brites, D. (2011). Bilirubin injury to neurons and glial cells: new players, novel targets, and newer insights. Semin Perinatol, 35(3), 114-120.

Brito, M. A., Brites, D., & Butterfield, D. A. (2004). A link between hyperbilirubinemia, oxidative stress and injury to neocortical synaptosomes. Brain Res, 1026(1), 33-43.

Brito, M. A., Lima, S., Fernandes, A., Falcão, A. S., Silva, R. F., Butterfield, D. A., et al. (2008a). Bilirubin injury to neurons: contribution of oxidative stress and rescue by glycoursodeoxycholic acid. Neurotoxicology, 29(2), 259-269.

Brito, M. A., Rosa, A. I., Falcão, A. S., Fernandes, A., Silva, R. F., Butterfield, D. A., et al. (2008b). Unconjugated bilirubin differentially affects the redox status of neuronal and astroglial cells. Neurobiol Dis, 29(1), 30-40.

Brito, M. A., Zurolo, E., Pereira, P., Barroso, C., Aronica, E., & Brites, D. (2011). Cerebellar axon/myelin loss, angiogenic sprouting and neuronal increase of vascular endothelial growth factor in a preterm infant with kernicterus. J Child Neurol, in press.

Calligaris, R., Bellarosa, C., Foti, R., Roncaglia, P., Giraudi, P., Krmac, H., et al. (2009). A transcriptome analysis identifies molecular effectors of unconjugated bilirubin in human neuroblastoma SH-SY5Y cells. BMC Genomics, 10, 543.

Carragher, N. O., & Frame, M. C. (2002). Calpain: a role in cell transformation and migration. Int J Biochem Cell Biol, 34(12), 1539-1543.

Casaccia-Bonnefil, P. (2000). Cell death in the oligodendrocyte lineage: a molecular perspective of life/death decisions in development and disease. Glia, 29(2), 124-135.

Chang, F. Y., Lee, C. C., Huang, C. C., & Hsu, K. S. (2009). Unconjugated bilirubin exposure impairs hippocampal long-term synaptic plasticity. PLoS One, 4(6), e5876.

Chen, H. C., Wang, C. H., Tsan, K. W., & Chen, Y. C. (1971). An electron microscopic and radioautographic study on experimental kernicterus. II. Bilirubin movement within neurons and release of waste products via astroglia. Am J Pathol, 64(1), 45-66.

Chen, L., Feng, X. C., Lu, F., Xu, X. L., Zhou, G. H., Li, Q. Y., et al. (2011). Effects of camptothecin, etoposide and Ca2+ on caspase-3 activity and myofibrillar disruption of chicken during postmortem ageing. Meat Sci, 87(3), 165-174.

Chen, Y., Balasubramaniyan, V., Peng, J., Hurlock, E. C., Tallquist, M., Li, J., et al. (2007). Isolation and culture of rat and mouse oligodendrocyte precursor cells. Nat Protoc, 2(5), 1044-1051.

Chew, L. J., Coley, W., Cheng, Y., & Gallo, V. (2010). Mechanisms of regulation of oligodendrocyte development by p38 mitogen-activated protein kinase. J Neurosci, 30(33), 11011-11027.

Claireaux, A. E. (2003). Pathology of human kernicterus. In A. Sass-Kortsak (Ed.), Kernicterus: A Report Based on a Symposium Held at the IX International Congress of Paediatrics (pp. 1961:1140). Toronto: University of Toronto Press.

Deganuto, M., Cesaratto, L., Bellarosa, C., Calligaris, R., Vilotti, S., Renzone, G., et al. (2010). A proteomic approach to the bilirubin-induced toxicity in neuronal cells reveals a protective function of DJ-1 protein. Proteomics, 10(8), 1645-1657.

Deng, W., Pleasure, J., & Pleasure, D. (2008). Progress in periventricular leukomalacia. Arch Neurol, 65(10), 1291-1295.

Deng, W., Rosenberg, P. A., Volpe, J. J., & Jensen, F. E. (2003). Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. Proc Natl Acad Sci U S A, 100(11), 6801-6806.

Falcão, A. S., Fernandes, A., Brito, M. A., Silva, R. F., & Brites, D. (2005). Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. Neurobiol Dis, 20(2), 199-206.

Falcão, A. S., Fernandes, A., Brito, M. A., Silva, R. F., & Brites, D. (2006). Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta Neuropathol, 112(1), 95-105.

Fernandes, A., Barateiro, A., Falcão, A. S., Silva, S. L., Vaz, A. R., Brito, M. A., et al. (2011). Astrocyte reactivity to unconjugated bilirubin requires TNF-alpha and IL-1beta receptor signaling pathways. Glia, 59(1), 14-25.

Fernandes, A., & Brites, D. (2009). Contribution of inflammatory processes to nerve cell toxicity by bilirubin and efficacy of potential therapeutic agents. Curr Pharm Des, 15(25), 2915-2926.

Fernandes, A., Falcão, A. S., Silva, R. F., Brito, M. A., & Brites, D. (2007). MAPKs are key players in mediating cytokine release and cell death induced by unconjugated bilirubin in cultured rat cortical astrocytes. Eur J Neurosci, 25(4), 1058-1068.

Fernandes, A., Silva, R. F., Falcão, A. S., Brito, M. A., & Brites, D. (2004). Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. J Neuroimmunol, 153(1-2), 64-75.

Fields, R. D., & Stevens-Graham, B. (2002). New insights into neuron-glia communication. Science, 298(5593), 556-562.

Franco, S., Perrin, B., & Huttenlocher, A. (2004). Isoform specific function of calpain 2 in regulating membrane protrusion. Exp Cell Res, 299(1), 179-187.

Fu, Y., Sun, W., Shi, Y., Shi, R., & Cheng, J. X. (2009). Glutamate excitotoxicity inflicts paranodal myelin splitting and retraction. PLoS One, 4(8), e6705.

Galluzzi, L., Blomgren, K., & Kroemer, G. (2009). Mitochondrial membrane permeabilization in neuronal injury. Nat Rev Neurosci, 10(7), 481-494.

Gard, A. L., & Pfeiffer, S. E. (1990). Two proliferative stages of the oligodendrocyte lineage (A2B5+O4- and O4+GalC-) under different mitogenic control. Neuron, 5(5), 615-625.

Genc, S., Genc, K., Kumral, A., Baskin, H., & Ozkan, H. (2003). Bilirubin is cytotoxic to rat oligodendrocytes in vitro. Brain Res, 985(2), 135-141.

Gkoltsiou, K., Tzoufi, M., Counsell, S., Rutherford, M., & Cowan, F. (2008). Serial brain MRI and ultrasound findings: relation to gestational age, bilirubin level, neonatal neurologic status and neurodevelopmental outcome in infants at risk of kernicterus. Early Hum Dev, 84(12), 829-838.

Goll, D. E., Thompson, V. F., Li, H., Wei, W., & Cong, J. (2003). The calpain system. Physiol Rev, 83(3), 731-801.

Gomes, A., Fernandes, E., & Lima, J. L. (2005). Fluorescence probes used for detection of reactive oxygen species. J Biochem Biophys Methods, 65(2-3), 45-80.

Gordo, A. C., Falcão, A. S., Fernandes, A., Brito, M. A., Silva, R. F., & Brites, D. (2006). Unconjugated bilirubin activates and damages microglia. J Neurosci Res, 84(1), 194-201.

Gu, C., Casaccia-Bonnefil, P., Srinivasan, A., & Chao, M. V. (1999). Oligodendrocyte apoptosis mediated by caspase activation. J Neurosci, 19(8), 3043-3049.

Gu, H., Chen, X., Gao, G., & Dong, H. (2008). Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. Mol Cancer Ther, 7(8), 2298-2307.

Gurba, P. E., & Zand, R. (1974). Bilirubin binding to myelin basic protein, histones and its inhibition in vitro of cerebellar protein synthesis. Biochem Biophys Res Commun, 58(4), 1142-1147.

Hansen, T., Tommarello, S., & Allen, J. (2001). Subcellular localization of bilirubin in rat brain after in vivo i.v. administration of [3H]bilirubin. Pediatr Res, 49(2), 203-207.

Hansen, T. W. R. (2000). Pioneers in the scientific study of neonatal jaundice and kernicterus. Pediatrics, 106(2), E15.

Jew, J. Y., & Williams, T. H. (1977). Ultrastructural aspects of bilirubin encephalopathy in cochlear nuclei of the Gunn rat. J Anat, 124(Pt 3), 599-614.

Kassmann, C. M., & Nave, K. A. (2008). Oligodendroglial impact on axonal function and survival - a hypothesis. Curr Opin Neurol, 21(3), 235-241.

Khorchid, A., Fragoso, G., Shore, G., & Almazan, G. (2002). Catecholamine-induced oligodendrocyte cell death in culture is developmentally regulated and involves free radical generation and differential activation of caspase-3. Glia, 40(3), 283-299.

Kim, R., Emi, M., Tanabe, K., & Murakami, S. (2006). Role of the unfolded protein response in cell death. Apoptosis, 11(1), 5-13.

Kotter, M. R., Stadelmann, C., & Hartung, H. P. (2011). Enhancing remyelination in disease--can we wrap it up? Brain, 134(Pt 7), 1882-1900.

Kraskiewicz, H., & FitzGerald, U. (2011). Partial XBP1 knockdown does not affect viability of oligodendrocyte precursor cells exposed to new models of hypoxia and ischemia in vitro. J Neurosci Res, 89(5), 661-673.

Kulkarni, P., Rajagopalan, K., Yeater, D., & Getzenberg, R. H. (2011). Protein folding and the order/disorder paradox. J Cell Biochem, 112(7), 1949-1952.

Lauer, B. J., & Spector, N. D. (2011). Hyperbilirubinemia in the newborn. Pediatr Rev, 32(8), 341-349.

Levine, J. M., Reynolds, R., & Fawcett, J. W. (2001). The oligodendrocyte precursor cell in health and disease. Trends Neurosci, 24(1), 39-47.

Li, J., & Lee, A. S. (2006). Stress induction of GRP78/BiP and its role in cancer. Curr Mol Med, 6(1), 45-54.

Limperopoulos, C. (2010). Advanced neuroimaging techniques: their role in the development of future fetal and neonatal neuroprotection. Semin Perinatol, 34(1), 93-101.

Lin, W., & Popko, B. (2009). Endoplasmic reticulum stress in disorders of myelinating cells. Nat Neurosci, 12(4), 379-385.

Liu, M. C., Kobeissy, F., Zheng, W., Zhang, Z., Hayes, R. L., & Wang, K. K. (2011). Dual vulnerability of tau to calpains and caspase-3 proteolysis under neurotoxic and neurodegenerative conditions. ASN Neuro, 3(1), e00051.

Lopes, J. P., Oliveira, C. R., & Agostinho, P. (2010). Neurodegeneration in an Abeta-induced model of Alzheimer's disease: the role of Cdk5. Aging Cell, 9(1), 64-77.

Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-Rosen, L. A., et al. (2000). Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. J Cell Biol, 149(3), 603-612.

Martich-Kriss, V., Kollias, S. S., & Ball, W. S., Jr. (1995). MR findings in kernicterus. AJNR Am J Neuroradiol, 16(4 Suppl), 819-821.

Mato, S., Victoria Sánchez-Gómez, M., & Matute, C. (2010). Cannabidiol induces intracellular calcium elevation and cytotoxicity in oligodendrocytes. Glia, 58(14), 1739-1747.

McDonagh, A. F., & Assisi, F. (1972). The ready isomerization of bilirubin IX- in aqueous solution. Biochem J, 129(3), 797-800.

McTigue, D. M., & Tripathi, R. B. (2008). The life, death, and replacement of oligodendrocytes in the adult CNS. J Neurochem, 107(1), 1-19.

Miller, R. H. (2002). Regulation of oligodendrocyte development in the vertebrate CNS. Prog Neurobiol, 67(6), 451-467.

Monge, M., Kadiiski, D., Jacque, C. M., & Zalc, B. (1986). Oligodendroglial expression and deposition of four major myelin constituents in the myelin sheath during development. An in vivo study. Dev Neurosci, 8(4), 222-235.

Mukhopadhyay, K., Chowdhary, G., Singh, P., Kumar, P., & Narang, A. (2010). Neurodevelopmental outcome of acute bilirubin encephalopathy. J Trop Pediatr, 56(5), 333-336.

Murakami, Y., Aizu-Yokota, E., Sonoda, Y., Ohta, S., & Kasahara, T. (2007). Suppression of endoplasmic reticulum stress-induced caspase activation and cell death by the overexpression of Bcl-xL or Bcl-2. J Biochem, 141(3), 401-410.

Nave, K. A. (2010). Myelination and support of axonal integrity by glia. Nature, 468(7321), 244-252.

Nishiyama, A., Lin, X. H., Giese, N., Heldin, C. H., & Stallcup, W. B. (1996). Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. J Neurosci Res, 43(3), 299-314.

Oakes, G. H., & Bend, J. R. (2010). Global changes in gene regulation demonstrate that unconjugated bilirubin is able to upregulate and activate select components of the endoplasmic reticulum stress response pathway. J Biochem Mol Toxicol, 24(2), 73-88.

Ozkan, H., Akkoc, N., Aydin, A., Kavukcu, Olgun, N., Irken, G., et al. (1995). Relationship between serum unconjugated bilirubin levels and the autofluorescence of white blood cells in neonatal jaundice. Biol Neonate, 68(2), 100-103.

Palmela, I., Cardoso, F. L., Bernas, M., Correia, L., Vaz, A. R., Silva, R. F., et al. (2011). Elevated levels of bilirubin and long-term exposure impair human brain microvascular endothelial cell integrity. Curr Neurovasc Res, 8(2), 153-169.

Panaretakis, T., Laane, E., Pokrovskaja, K., Bjorklund, A. C., Moustakas, A., Zhivotovsky, B., et al. (2005). Doxorubicin requires the sequential activation of caspase-2, protein kinase Cdelta, and c-Jun NH2-terminal kinase to induce apoptosis. Mol Biol Cell, 16(8), 3821-3831.

Pfeiffer, S. E., Warrington, A. E., & Bansal, R. (1993). The oligodendrocyte and its many cellular processes. Trends Cell Biol, 3(6), 191-197.

Pirianov, G., Jesurasa, A., & Mehmet, H. (2006). Developmentally regulated changes in c-Jun N-terminal kinase signalling determine the apoptotic response of oligodendrocyte lineage cells. Cell Death Differ, 13(3), 531-533.

Romisch, K. (2005). Endoplasmic reticulum-associated degradation. Annu Rev Cell Dev Biol, 21, 435-456.

Ruiz, A., Matute, C., & Alberdi, E. (2010). Intracellular Ca2+ release through ryanodine receptors contributes to AMPA receptor-mediated mitochondrial dysfunction and ER stress in oligodendrocytes. Cell Death Dis, 1, e54.

Rutkowski, D. T., & Kaufman, R. J. (2004). A trip to the ER: coping with stress. Trends Cell Biol, 14(1), 20-28.

Saatman, K. E., Creed, J., & Raghupathi, R. (2010). Calpain as a therapeutic target in traumatic brain injury. Neurotherapeutics, 7(1), 31-42.

Sánchez-Gómez, M. V., Alberdi, E., Ibarretxe, G., Torre, I., & Matute, C. (2003). Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors. J Neurosci, 23(29), 9519-9528.

Sánchez-Gómez, M. V., Alberdi, E., Perez-Navarro, E., Alberch, J., & Matute, C. (2011). Bax and calpain mediate excitotoxic oligodendrocyte death induced by activation of both AMPA and kainate receptors. J Neurosci, 31(8), 2996-3006.

Shapiro, S. M. (2010). Chronic bilirubin encephalopathy: diagnosis and outcome. Semin Fetal Neonatal Med, 15(3), 157-163.

Silberberg, D. H., & Schutta, H. S. (1967). The effects of unconjugated bilirubin and related pigments on cultures of rat cerebellum. J Neuropathol Exp Neurol, 26(4), 572-583.

Silva, R., Mata, L. R., Gulbenkian, S., Brito, M. A., Tiribelli, C., & Brites, D. (1999). Inhibition of glutamate uptake by unconjugated bilirubin in cultured cortical rat astrocytes: role of concentration and pH. Biochem Biophys Res Commun, 265(1), 67-72.

Silva, R. F., Rodrigues, C. M., & Brites, D. (2001). Bilirubin-induced apoptosis in cultured rat neural cells is aggravated by chenodeoxycholic acid but prevented by ursodeoxycholic acid. J Hepatol, 34(3), 402-408.

Silva, S. L., Vaz, A. R., Barateiro, A., Falcão, A. S., Fernandes, A., Brito, M. A., et al. (2010). Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. Neurobiol Dis, 40(3), 663-675.

Sorimachi, H., Ishiura, S., & Suzuki, K. (1997). Structure and physiological function of calpains. Biochem J, 328 (Pt 3), 721-732.

Upton, J. P., Austgen, K., Nishino, M., Coakley, K. M., Hagen, A., Han, D., et al. (2008). Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. Mol Cell Biol, 28(12), 3943-3951.

Vaz, A. R., Delgado-Esteban, M., Brito, M. A., Bolaños, J. P., Brites, D., & Almeida, A. (2010). Bilirubin selectively inhibits cytochrome c oxidase activity and induces apoptosis in immature cortical neurons: assessment of the protective effects of glycoursodeoxycholic acid. J Neurochem, 112(1), 56-65.

Vaz, A. R., Silva, S. L., Barateiro, A., Falcão, A. S., Fernandes, A., Brito, M. A., et al. (2011a). Selective vulnerability of rat brain regions to unconjugated bilirubin. Mol Cell Neurosci. 48(1), 82-93.

Vaz, A. R., Silva, S. L., Barateiro, A., Fernandes, A., Falcão, A. S., Brito, M. A., et al. (2011b). Pro-inflammatory cytokines intensify the activation of NO/NOS, JNK1/2 and caspase cascades in immature neurons exposed to elevated levels of unconjugated bilirubin. Exp Neurol, 229(2), 381-390.

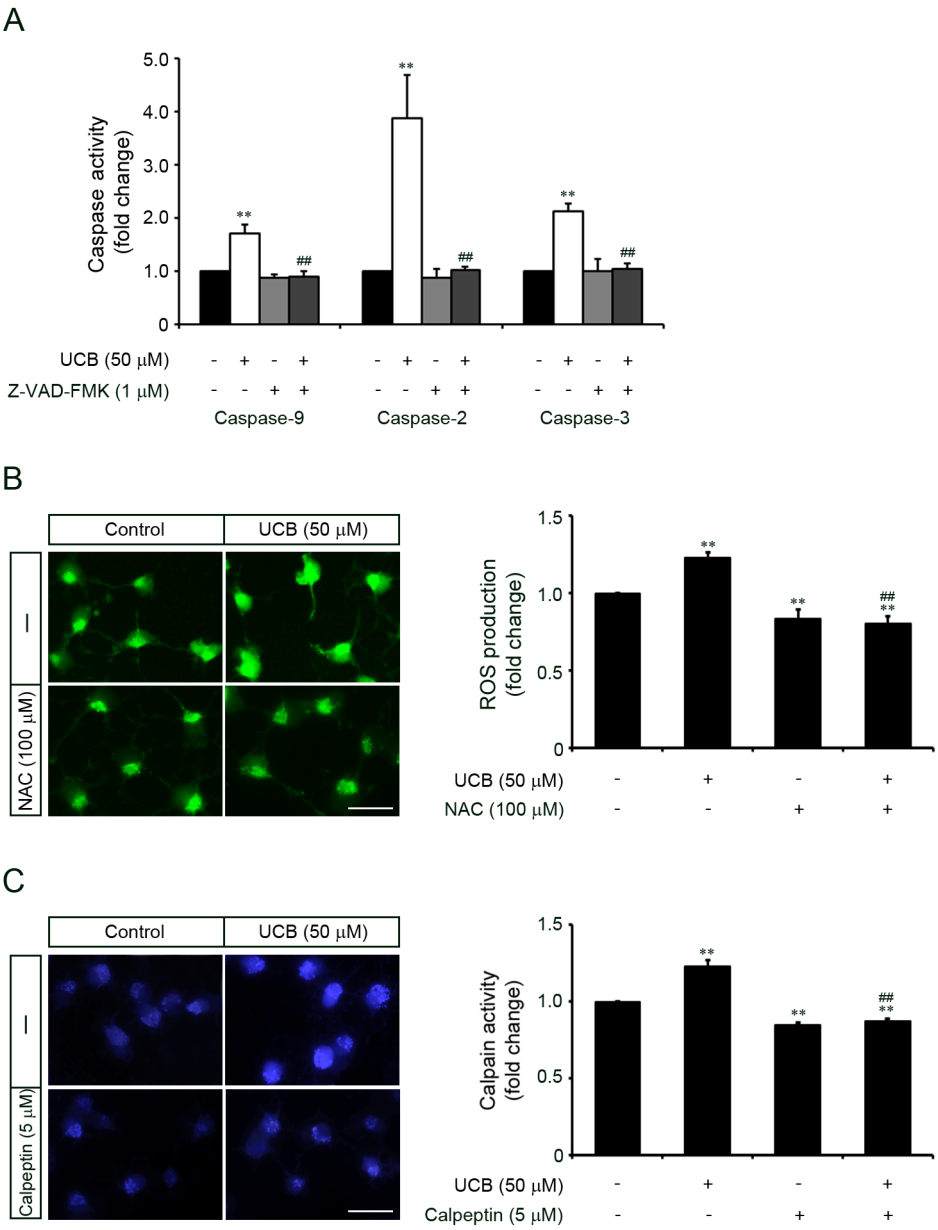
Verma, G., & Datta, M. (2011). The critical role of JNK in the ER-mitochondrial crosstalk during apoptotic cell death. J Cell Physiol.

Waldbaum, S., & Patel, M. (2010). Mitochondria, oxidative stress, and temporal lobe epilepsy. Epilepsy Res, 88(1), 23-45.

Wu, J., & Kaufman, R. J. (2006). From acute ER stress to physiological roles of the Unfolded Protein Response. Cell Death Differ, 13(3), 374-384.

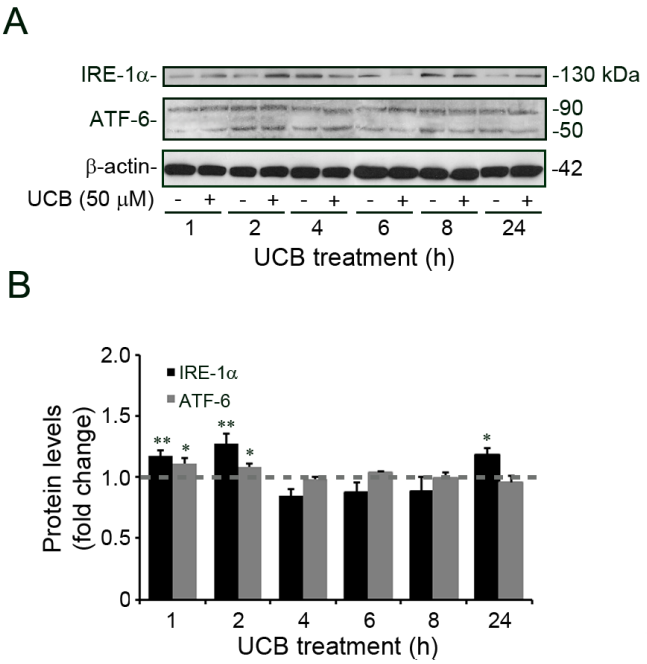
6. Supplementary Figures

6.1. Supplementary Figure II.1



Suppl. Fig. II.1 – Inhibition of caspases, calpains and oxidative stress prevented unconjugated bilirubin (UCB)-induced oligodendrocyte precursor cell (OPC) death. Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin and to specific inhibitor for caspases (Z-VAD-FMK, 1 μM), calpains (calpeptin, 25 μM) or oxidative stress (N-acetylcysteine - NAC, 100 μM). **a** Caspase-9, -2 and -3 activities with or without Z-VAD-FMK incubation were determined by colorimetric substrate cleavage assay as indicated in Materials and methods at 4, 8 and 24 h, respectively. Graph bars represent the fold change from vehicle (mean ± SEM), from at least three independent experiments. **b** Reactive oxygen species (ROS) were determined at 6 h by fluorescence assay with dihydrorhodamine 123 as described in Material and methods after incubation in the presence or absence of NAC. Representative images of one experiment and graph bars with fold change from vehicle (mean ± SEM) in ROS production from at least three independent experiments are shown. **c** Calpain activity was determined at 4 h of incubation using a specific fluorogenic substrate as indicated in Material and methods after treatment with or without calpeptin. Representative images of one experiment and graph bars with fold change from vehicle (mean ± SEM) for calpain activation from at least three independent experiments are shown. $^{**}P<0.01$ vs. respective vehicle; $^{\#\#}P<0.01$ vs. respective UCB.

6.2. Supplementary Figure II.2



Suppl. Fig. II.2 – Unconjugated bilirubin (UCB) induces an increase in activating transcription factor 6 (ATF-6) and inositol-requiring enzyme 1α (IRE-1α), chaperones usually involved in endoplasmic reticulum stress, in oligodendrocyte precursor cells (OPC). Rat cortical OPC were exposed to 50 μM UCB in the presence of 100 μM human serum albumin for the indicated time periods. **a** Representative results of ATF-6 and IRE-1α protein expression by Western blot using specific antibodies are shown. **b** Graph bars represent the fold increase from vehicle (mean ± SEM) obtained for protein band intensity by scanning densitometry standardized with respect to β-actin protein for ATF-6 and IRE-1α determined as indicated in Materials and methods, from at least four independent experiments. ***P*<0.01 and **P*<0.05 vs. respective vehicle.

Chapter III

ARREST OF OLIGODENDROCYTE DIFFERENTIATION AND AXONAL MYELINATION IN AN *IN VITRO* MODEL OF HYPERBILIRUBINEMIA

Andreia Barateiro[1], Veronique E. Miron[2], Sofia D. Santos[3], João B. Relvas[3,4], Adelaide Fernandes[1,5], Charles ffrench-Constant[2], Dora Brites[1,5]

[1]Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculty of Pharmacy, University of Lisbon, Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

[2]MRC Centre for Regenerative Medicine
Scottish Centre for Regenerative Medicine, The University of Edinburgh
5 Little France Drive, Edinburgh, EH16 4UU, United Kingdom

[3]Instituto de Biologia Molecular e Celular, University of Porto
Rua do Campo Alegre 823, 4150-180 Porto, Portugal

[4]Departamento de Biologia Experimental, Faculty of Medicine, University of Porto
Av. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

[5]Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

Glia, 2012 (submitted)

Acknowledgments

This work was supported by the strategic project PEst-OE/SAU/UI4013/2011 and PTDC/SAU-NEU/64385/2006 grants from Fundação para a Ciência e a Tecnologia (FCT), Lisbon, Portugal (to D. B.). A. B. was recipient of a PhD fellowship (SFRH/BD/43885/2008) from FCT. SDS acknowledges POPH - QREN-Tipologia 4.2 - Promotion of scientific employment funded by the ESF and MCTES. Work in the lab of JBR was supported by a PTDC/BIA-BCM/112730/2009 from FCT. V.M. received a post-doctoral fellowship from the Multiple Sclerosis Society of Canada. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

High levels of serum unconjugated bilirubin (UCB) in newborn children are associated with axonal damage and glial reactivity which may account for subsequent neurologic impairment and changes in brain white matter. We recently reported that UCB promotes oligodendrocyte progenitor cell (OPC) death. We hypothesized that neonatal hyperbilirubinemia may also influence oligodendrocyte (OL) maturation and myelination. Using primary cultures of OL, we show that UCB exposure prior to the onset of differentiation inhibited OL maturation resulting in higher numbers of OPC and lower numbers of mature OL compared to control cells. This was associated with significant downregulation of Olig1 and upregulation of Olig2 mRNA levels. UCB treatment prior to or during differentiation impaired OL morphological maturation, processes extension, and cell diameter. Both experimental paradigms caused a reduction in the active guanosine triphosphate (GTP)-bound Rac1 fraction. In myelinating co-cultures of dorsal root ganglia neurons and OL, UCB treatment prior to the onset of myelination decreased oligodendroglial differentiation and number of myelinating OL, the latter was also observed upon UCB exposure once myelination was already initiated. Both treatments also decreased the number of myelin internodes per OL and myelin internode length. Taken together, these observations show a deleterious role of UCB in myelinogenesis, which may compromise axonal function and transmission of the nerve impulse.

Keywords: Oligodendrocytes; myelinating co-cultures; myelin deficits; unconjugated bilirubin

1. Introduction

Myelin sheath formation in the brain occurs predominantly postnatally and is of critical importance to vertebrate development. Myelination is necessary for axon survival and permits a rapid impulse conduction of action potentials. On the other hand, the survival of oligodendrocytes (OL), the myelinating glial cells in the central nervous system (CNS), depends on their interactions with axons. In the CNS, OL arise from precursors of the ventricular and subventricular zones (Miller 1996) and migrate into the brain parenchyma (Goldman 1995). Postnatally, these cells cease to migrate, become postmitotic, and undergo differentiation from NG2 proteoglycan+ immature oligodendrocyte progenitor cells (OPC) to O4+ preoligodendrocytes and then myelin basic protein (MBP)+ mature myelinating OL (Miller 2002).

During OL development, OPC are initially less ramified then upon differentiation to immature OL, extend a complex network of processes towards multiple axons and initiate the expression of myelin components. Once axoglial contact has been established, mature OL produce large amounts of specialized membrane that form multiple wraps around the contacted axons with subsequent compaction of this multispiral membrane forming myelin (Bunge 1968). Small GTPases of the Rho family, like cdc42, Rac1 and RhoA, are signalling molecules that regulate the organization of the actin cytoskeleton, microtubule dynamics, cell polarity and membrane trafficking in response to environmental signals (Etienne-Manneville and Hall 2002). Rac1 promotes actin polymerization to produce lamellipodia and ruffles, whereas RhoA leads to the assembly of stress fibers and of focal adhesions (Nobes and Hall 1995).

The cells committed to the oligodendroglial lineage are highly responsive to multiple extracellular factors, which regulate their migration, proliferation and differentiation. OPC are dependent on the presence of fibroblast growth factor 2 (FGF-2) and platelet derived growth factor (PDGF) to maintain a proliferating phenotype, and as so can be kept *in vitro* in a proliferative state for extended periods of time with a combination of these two growth factors (Bogler et al. 1990). On the other hand, thyroid hormone (T3) appears to be required for OPC to exit the cell cycle and differentiate into mature OL (Barres et al. 1994), being used *in vitro* to promote OPC differentiation. However, regulation of OL pathway is multifactorial, and the transcription factors Olig1 and Olig2 also play specific roles during OPC differentiation (Ligon et al. 2006). Absence of Olig1 signalling leads to OL arrest at an early stage of differentiation (Xin et al. 2005), while Olig2 is required for specification to the OL lineage (Copray et al. 2006) and is always expressed in the nucleus, with higher levels in OPC relative to mature OL (Kitada and Rowitch 2006).

Perhaps, due to this complex differentiation program, and their unique metabolism, OL account for one of the most vulnerable cells of the CNS. Perturbations in myelination during the perinatal period will consequently result in axon dysfunction and abnormal electrical conduction, therefore impairing the transfer of information across brain regions and contributing to brain dysfunction and long-term neurological impairments (Dewar et al. 2003).

In newborns, bilirubin concentrations are frequently increased (Rubaltelli and Griffith 1992) and significant health hazards have been linked to significant hyperbilirubinemia, mainly in premature infants (Lauer and Spector 2011). Physiological to pathological transition is driven by the deposition of unconjugated bilirubin (UCB) in the brain leading to either acute or chronic encephalopathy, the latter also termed *kernicterus* (Hansen 2002). Brain sections of icteric infants show UCB within neurons, neuronal processes and microglia (Hansen 2000), in parallel with neuronal loss, astrogliosis and bilirubin-lipid complexes phagocytosed by macrophages within foci of periventricular white matter necrosis (Ahdab-Barmada and Moossy 1984). A recent study reported loss of myelin fibers in cerebellar sections from a pre-term infant with *kernicterus* (Brito et al. 2011). Moreover, reduction of white matter volume and delay in hemispheric myelination determined by magnetic resonance imaging was observed in infants at risk of bilirubin-induced neurological dysfunction (BIND) (Gkoltsiou et al. 2008). Other studies showed that UCB affects myelination in tissue cultures of rat cerebellum (Silberberg and Schutta 1967), and has ability to bind to MBP (Gurba and Zand 1974), appearing in high concentrations in the myelin fraction after injection into rats (Hansen et al. 2001).

In vitro studies have similarly demonstrated that UCB induces several alterations in different types of nerve cells, including oxidative stress, glutamate release, long-lasting impairment of neuritic arborization (Brites 2011). Our recent study has shown that UCB reduces OPC survival following endoplasmic reticulum (ER) stress, mitochondrial dysfunction, oxidative stress and activation of caspases and calpains (Barateiro et al. 2012). UCB has been previously shown to decrease mature OL viability in a concentration and time-dependent manner and increase nitric oxide synthase (NOS) mRNA expression (Genc et al. 2003). Given that the effects of UCB on OPC maturation into mature OL and their ability to myelinate have never been described, we tested the effect of UCB exposure on *in vitro* models of differentiation and myelination.

Here, we show that UCB impairs OL differentiation, by reducing the transition of OPC to mature OL, resulting in an enhanced number of OPC and concomitantly a decreased number of mature OL, while it also decreased mature OL diameter and

branching complexity. Moreover, using a myelinating co-culture system, we observed that UCB decreases OPC maturation, reduces the number of myelinated internodes per mature OL, and decreases internode length. Collectively, our data suggests that UCB compromises oligodendrogenesis and myelination, thus impairing axonal function and transmission of the nerve impulse. Of note, UCB effects are more pronounced if exposure occurs in earlier stages of OPC maturation and prior to the onset of myelination, thereby accounting for the known proneness of premature newborns to BIND.

2. Material and Methods

2.1. Reagents and media

Dulbecco's modified Eagle's medium (DMEM), 50 U/mL penicillin/50 µg/mL streptomycin, GlutaMAX™, normal horse serum (NHS) and fetal calf serum (FCS) were purchased from Gibco (Life Technologies, Inc., Grand Islands, USA). L-cysteine hydrochloride, 5-fluoro-2′-deoxyuridine (FdU), deoxyribonuclease I type 4 (DNase I), L-glutamine, putrescine, L-thyroxine (T4), tri-iodothyroxine (T3), progesterone, sodium selenite, bovine serum albumin fraction V (BSA), insulin, holo-transferrin, ITS liquid media supplement (100x) and human serum albumin (HSA) (fraction V, fatty acid free) were acquired from Sigma (St. Louis, MO, USA). UCB was also obtained from Sigma and purified according to the method of McDonagh (McDonagh and Assisi 1972). Papain was obtained from Worthington (Lakewood, NJ, USA). Mouse nerve growth factor 2.5S (NGF) and rat antibody anti-MBP were from AbD Serotec (#MCA409S, Raleigh, NC, USA). BD Matrigel™ basement membrane matrix growth factor reduced was from BD Biosciences (Erembodegem, Belgium). Human platelet-derived growth factor-AA (PDGF-AA) and basic fibroblast growth factor (bFGF) were obtained from PeproTech (Rocky Hill, NJ). Rabbit antibody anti-NG2 (#AB5320), mouse antibody anti-Rho (#05-778) and mouse antibody anti-Rac1 (#05-389) were purchased from Millipore (Billerica, MA, USA). Chicken antibody anti-neurofilament was acquired from Covance (Princeton, NJ, USA). Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-rat IgG, Alexa Fluor 488 goat anti-chicken IgG, Alexa Fluor 555 goat anti-rat IgG and Alexa Fluor 647 donkey anti-rabitt were purchased from Life Sciences (Carlsbad, CA, USA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Thermo Scientific (Waltham, MA, USA). DNase I and SuperScript II Reverse Transcriptase were acquired from Invitrogen (Carlsbad, CA, USA). Quick-RNA™ MicroPrep was obtained from Zymo

Research (Irvine, CA, USA). SYBR Green qPCR Master Mix was purchased from BioRad (Hercules, CA, USA).

2.2. Animals

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

2.3. Oligodendrocyte cell culture and treatment

Primary OPC cultures were harvested from post-natal day (P) 1 to P2 neonatal Wistar rat cortex following a standard protocol (McCarthy and de Vellis 1980) with minor modifications (Syed et al. 2008). In brief, dissociated rat neonatal cortices were cultured at 37 °C in 7.5% CO_2 in DMEM with 10% FCS and penicillin/streptomycin for 10 days *in vitro* (DIV). To remove the microglia on the top of the mixed glial cultures, the flasks were shaken for 1 h at 260 r.p.m. on an orbital shaker before being shaken at 260 r.p.m. overnight to dislodge the loosely attached OPC. These OPC were further purified from contaminating microglia by a differential adhesion step. Purified OPC were plated at a density of 20,000 cells per 0.8 cm^2 well in SATO media [L-glutamine (4 mM), putrescine (16 µg/mL), T4 (400 µg/mL), T3 (400 µg/mL), progesterone (6.2 ng/mL), sodium selenite (5 ng/mL), BSA V (100 µg/mL), insulin (5 µg/mL), holo-transferrin (50 µg/mL)] supplemented with PDGF-AA (10 ng/mL), FGF-2 (10 ng/mL), 1% penicillin/streptomycin, 1% ITS and 0.5% FCS. After 1 DIV, to induce differentiation, PDGF-AA and FGF were replaced by 3% FCS in SATO medium and cells kept in culture for 7 DIV in order to obtain an enriched culture of mature OL.

To evaluate if UCB treatment during the proliferation vs. the differentiation stages would affect OL maturation, OPC cultures were incubated with 50 µM UCB plus 100 µM HSA, or only with 100 µM HSA (vehicle), either during the first 24 h after OPC isolation, when cells are cultured with PDGF-AA and FGF-2, or during the first 24 h after the induction of differentiation with FCS. All UCB solutions were prepared from 10 mM stock solution in 0.1 N NaOH immediately before use and the pH of the incubation media was restored to 7.4 by addition of equal amounts of 0.1 N HCl. We

have consistently used this stable solution of UCB:HSA=0.5 for several years to avoid UCB precipitation (Brites 2011). Moreover, by always using the same experimental model of hyperbilirubinemia we can compare susceptibilities between nerve cells to UCB. All the experiments with UCB were performed under light protection to avoid photodegradation.

2.4. Myelinating co-culture and treatment

This culture system was obtained in accordance to the method described by Laursen et al. (Laursen et al. 2009), with minor modifications. Briefly, dorsal root ganglia (DRG) neurons were isolated from embryonic day 15 Sprague-Dawley rats and dissociated with papain (1.2 U/mL), L-cysteine (0.24 mg/mL) and DNase (0.4 mg/mL) for 60 min at 37ºC. After dissociation, neurons were plated at a density of 150,000 cells per coverslip (18 mm) coated with poly-D-lysine and growth factor-reduced Matrigel, and cultured for 21 DIV in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and NGF (100 ng/mL). In order to remove contaminating cells, the culture was pulsed three times for 48 h with fluorodeoxyuridine at 1, 4 and 7 DIV after seeding. After 21 DIV the medium was changed to basal media Eagle supplemented with 1% ITS, 1% penicillin/streptomycin, 1% glutamax, 1% glucose (45% solution) and 0.5% FCS. At this time OPC generated as described above were seeded onto the neurons at a density of 75,000 per coverslip. Co-cultures were maintained for 15 DIV with media changes every 2-3 DIV.

The co-culture system was then treated with UCB as described above, at 1 and 7 days of co-culture (DCC), in order to evaluate the effects produced by UCB either prior to the beginning of myelination or during the myelination process, respectively.

2.5. Immunocytochemistry and data analysis of primary oligodendrocyte cell culture

Primary OL cultures were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered solution (PBS) for 15 min and then permeabilized with PBS containing 1% HEPES buffer, 2.5% Triton X-100, 1% goat serum and 1% normal horse serum. The cultures were immunolabelled with primary antibodies against the different stages of OL differentiation, e.g. NG2 for OPC and MBP for mature OL for 1 h at room temperature. Cells were incubated with secondary antibodies conjugated to Alexa Fluor 568 and Alexa Fluor 488, after rinsing the cells in PBS. Photomicrographs were by confocal microscopy (Leica SPE, Leica Microsystems, Cambridge, England). The

number of NG2+ and MBP+ cells were counted in a minimum of 200 Hoescht+ nuclei from at least four independent experiments and expressed as percentage vs. total cell number. To categorize the morphology of MBP+ cells, three different stages were defined: cells with poorly branched processes (stage 1), cells with complex branched processes (stage 2) and cells with complex branched processes that partially form membranes (stage 3). Diameter of MBP+ OL was measured with ImageJ software. To quantify the morphological complexity of the processes, first principal processes of interest were outlined using NeuronJ for ImageJ to exclude adjacent cells. Subsequently, a minimum of 50 MBP+ cells from each condition were analysed for the number of intersections made by branching processes with each successive circle using Sholl analysis for ImageJ. The program superimposes a grid of 20 concentric circles with increasing radius on an OL cell body (starting radius, 4 μm; step size, 4 μm; end, 80 μm).

2.6. Quantitative PCR

Total RNA was extracted from maturing OL after 7 DIV, treated as described before, using Quick-RNA™ MicroPrep Kit, according to manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 1 μg of total RNA were treated with DNase I and then reverse transcribed cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase under the recommended conditions. Quantitative RT-PCR (qRT-PCR) was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control to normalize the expression level of Olig1 and Olig2 transcription factors. The following sequences were used as primers: Olig1 sense 5'-GCCCAGGCCACGAGTACAAA-3' and anti-sense 5'-TCCACTCCGAAACCCAACGA-3' (Labombarda et al. 2009); Olig2 sense 5'-GAAATGGAATAATCCCGAACTACT-3' and anti-sense 5'-CCCCTCCCAAATAACTCAAAC-3' (Labombarda et al. 2009); GAPDH sense 5'-TGGAGTCTACTGGCGTCTT-3' and anti-sense 5'-TGTCATATTTCTCGTGGTTCA-3'. qRT-PCR was performed on a iQ5 (BioRad) using a SYBR Green qPCR Master Mix. The PCR was performed in 96 well plates with each sample performed in triplicate, and no-template control was included for each amplificate. qRT-PCR was performed under optimized conditions: 94ºC at 3 min followed by 40 cycles at 94ºC for 0.15 min, 60ºC for 0.2 min and 72ºC for 0.15 min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. Relative mRNA

concentrations were calculated using the Pfaffl modification of the $\Delta\Delta C_T$ equation (C_T, cycle number at which fluorescence passes the threshold level of detection), taking into account the efficiencies of individual genes. The results were normalized to GAPDH in the same sample and the initial amount of the template of each sample was determined as relative expression by the formula $2^{-\Delta\Delta CT}$. ΔC_T is a value obtained, for each sample, by the difference between the mean C_T value of each Olig gene and the mean C_T value of GAPDH. $\Delta\Delta C_T$ of one sample is the difference between its ΔC_T value and ΔC_T of the sample chosen as reference, in our case the vehicle-treated cells.

2.7. GTPase Pull-Down Assays

To detect active Rho and Rac in cultured OL we performed pull-down assays. The Rac bait protein (GST-p21-activated kinase-crib domain) and the Rho bait protein (GST-Rhotekin) were produced in BL21 *Escherichia coli*. The GST-tagged protein was bound to the glutathione-coupled Sepharose beads at 4ºC for 30 minutes. The concentration of $4.5x10^6$ rat OPC were seeded as described previously, lysed in FISH buffer (10% glycerol, 50 mM Tris pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$, 0.005% Triton X-100 and protease inhibitor cocktail) and centrifuged at 21,000 g for 5 min. To determine the pull-down input, 10% of the supernatant was removed. The remaining supernatant was incubated during 45 min, at 4ºC, with bait proteins bound to glutathione-coupled Sepharose beads previously prepared. Bound guanosine triphosphate (GTP) proteins were eluted with a column after boiling at 95ºC for 10 min, separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis together with the input and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk and immunoblotted with primary antibodies overnight at 4ºC [mouse anti-Rho (1:1,500) and mouse anti-Rac1 (1:1,000)] and then with peroxidase-conjugated secondary antibodies for 1 h at room temperature. After extensive washes, immunoreactive bands were detected with SuperSignal West Pico Chemiluminescent Substrate and visualized by autoradiography. The relative intensities of protein bands were analysed using Quantity One (version 4.6) program (Bio-Rad, Hercules, CA, USA). Results were expressed as percentage of activated protein in relation to total protein (pull down input).

2.8. Immunocytochemistry and data analysis of myelinating co-culture

To evaluate changes in myelination after UCB exposure, cells were fixed and permeabilized as described above. Changes in culture composition were assessed by

immunocytochemical staining using specific primary antibodies for neurofilaments, OPC (NG2) and mature OL (MBP), followed by detection with secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 647 and Alexa Fluor 555, respectively. Seven random pictures were acquired of areas with minimum 70% axonal coverage. The number of NG2 and MBP positive cells were counted and expressed as percentage of positive cells vs. total number of OL (NG2 plus MBP). MBP+ cells were then categorised in three different stages of maturation: cells that only extend processes but do not contact neurofilaments, cells that establish contact with neurofilaments but do not myelinate and cells that wrap axons having at least two internodes connected to the cell body. An internode was defined as a tube of MBP staining surrounding an axon. We evaluated the number of myelinated internodes formed by a single OL and the internode length was manually traced and measured on ImageJ.

In addition, in order to verify if UCB exposure induces axonal damage, we have determined the neurofilaments area. Briefly, binary masks were defined using the same cut-off intensity threshold value for each region of interest, defined as the minimum intensity due to specific staining above background values. Then, the percentage of the area occupied by neurofilaments was measured automatically using ImageJ software in each field by averaging values determined in at least five separate microscopic fields. Values are expressed as mean ± SEM.

2.9. Statistical analysis

All results are presented as mean ± SEM. Significant differences between two groups were determined by the two-tailed *t*-test performed on the basis of equal and unequal variance as appropriate and the P-values of $*P<0.05$ and $**P<0.01$ were considered as statistically significant.

3. Results

3.1. UCB impairs the differentiation of oligodendrocyte progenitors into myelinating cells

In order to evaluate the effect of UCB in OL maturation, primary OPC derived from neonatal rats were exposed to UCB during proliferation, e.g. first 24 h after seeding, 1st DIV, or upon the first 24 h of differentiation, 2nd DIV. After 7 DIV in differentiation medium, cells were immunostained for NG2 and MBP to calculate the number of OPC and mature OL, respectively. As demonstrated in Fig. III.1, UCB treatment during proliferation impaired the differentiation process resulting in a significant reduction of mature MBP+ cells (0.86±0.02 fold, $P<0.01$) in favour of an increase of immature NG2+ cells (2.75±0.32 fold, $P<0.01$). When OPC treatment with UCB occurred during initial differentiation, the same trend effect by UCB was observed although no significant results were obtained (Fig. III.1b).

Taking into account that Olig1 and Olig2 transcription factors modulate OL function under physiological and pathological circumstances (Ligon et al. 2006; Lin et al. 2006), their measurement can, therefore, provide an index of how UCB may modulate early and late steps of maturation. Fig. III.1c shows the change in the mRNA expression of the transcription factors Olig1 and Olig2 in vehicle- and UCB-treated cells during proliferation and differentiation. After 7 DIV, treatment with UCB during proliferation triggered a significant reduction in Olig1 and a significant increase in Olig2 mRNA levels (0.65±0.15 fold and 1.93±0.33 fold, $P<0.05$ and $P<0.01$, respectively). Treatment during differentiation also produced a significant increase in Olig 2 mRNA levels (1.70±0.28 fold, $P<0.01$).

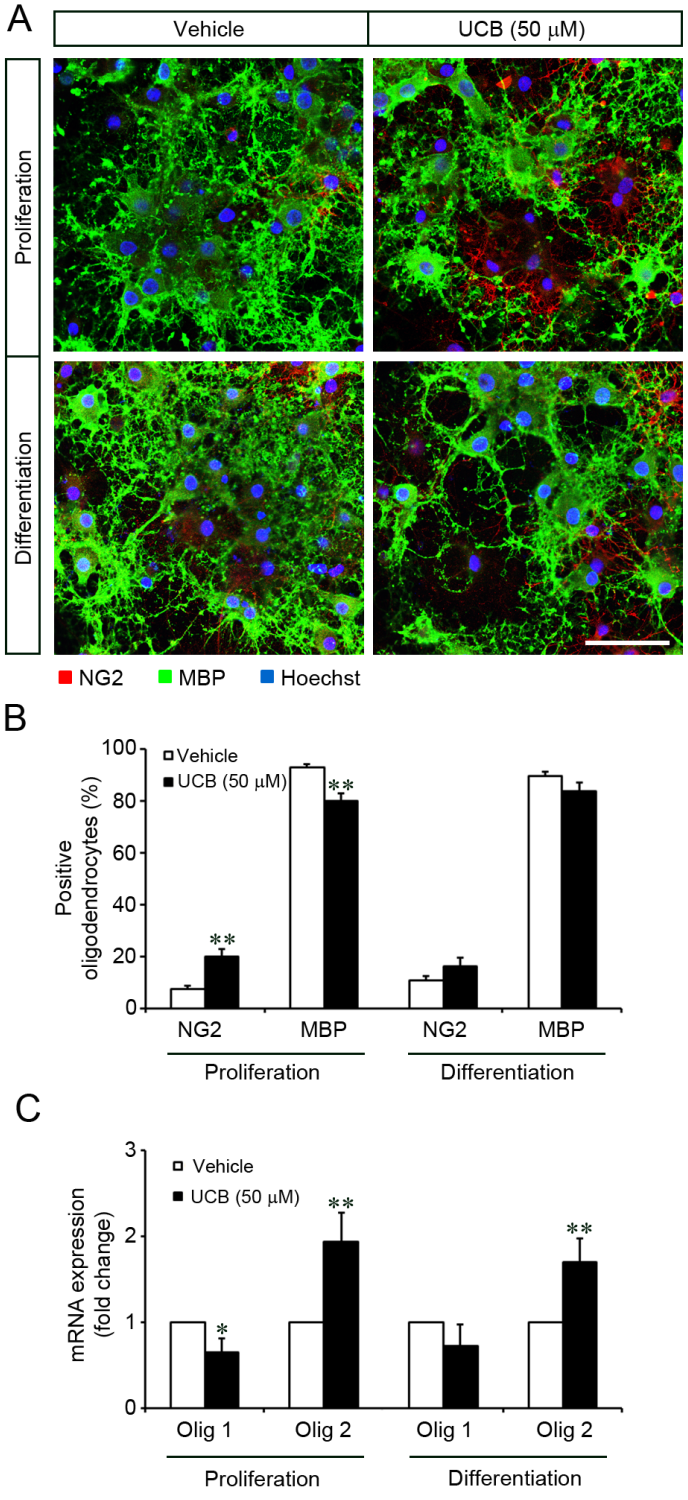


Fig. III.1 – Unconjugated bilirubin (UCB) impairs oligodendrocyte maturation if exposure occurs during the proliferation stage. Rat cortical oligodendrocytes were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during the 24 h of proliferation or during the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte maturation. After 7 days *in vitro* oligodendrocytes were immunostained to identify oligodendrocyte precursor cells that express NG2 marker (red) and mature oligodendrocytes that express myelin basic protein (MBP) (green). Nuclei were counterstained with Hoechst dye (blue). **a** Representative images of oligodendrocytes exposed to UCB during proliferation (top row) or during the first 24 h of differentiation (bottom row). **b** Quantification of the relative numbers of NG2+ and MBP+ cells following different UCB treatments, from at least six independent experiments performed in duplicate. **c** Relative levels of transcription factors Olig1 and Olig2 determined by qRT-PCR with the $\Delta\Delta C_T$ method. Values are shown as mean ± SEM from at least four independent experiments performed in triplicate, normalized to values measured for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same samples. Scale bar represents 50 μm. ***P*<0.01 vs. respective vehicle.

3.2. UCB prevents oligodendrocyte processes extension, myelin membrane formation and impairs Rac activation

Besides the expression of myelin components such as MBP, the morphologic complexity and the formation of myelin membranes are important features of terminal OL differentiation.

In line with the effect of UCB on OL maturation, the presence of UCB during proliferation produced a decrease in the complexity of cell processes, increasing the numbers of mature OL in stage 1 (1.79±0.22 fold, P<0.01) and decreasing them in stages 2 and 3 (0.83±0.01 fold and 0.32±0.04 fold, respectively, P<0.01) (Fig. III.2a). Interestingly, UCB exposure during differentiation produced a very similar effect, with an increase in stage 1 mature OL (1.74±0.17 fold, P<0.01) and a decrease in stages 2 and 3 (0.84±0.03 fold and 0.36±0.12 fold respectively, P<0.01) (Fig. III.2a). As depicted in Fig. III.2b, the cell diameter was decreased after UCB treatment during proliferation and differentiation (0.78±0.03 fold and 0.82±0.03 fold, respectively, P<0.01).

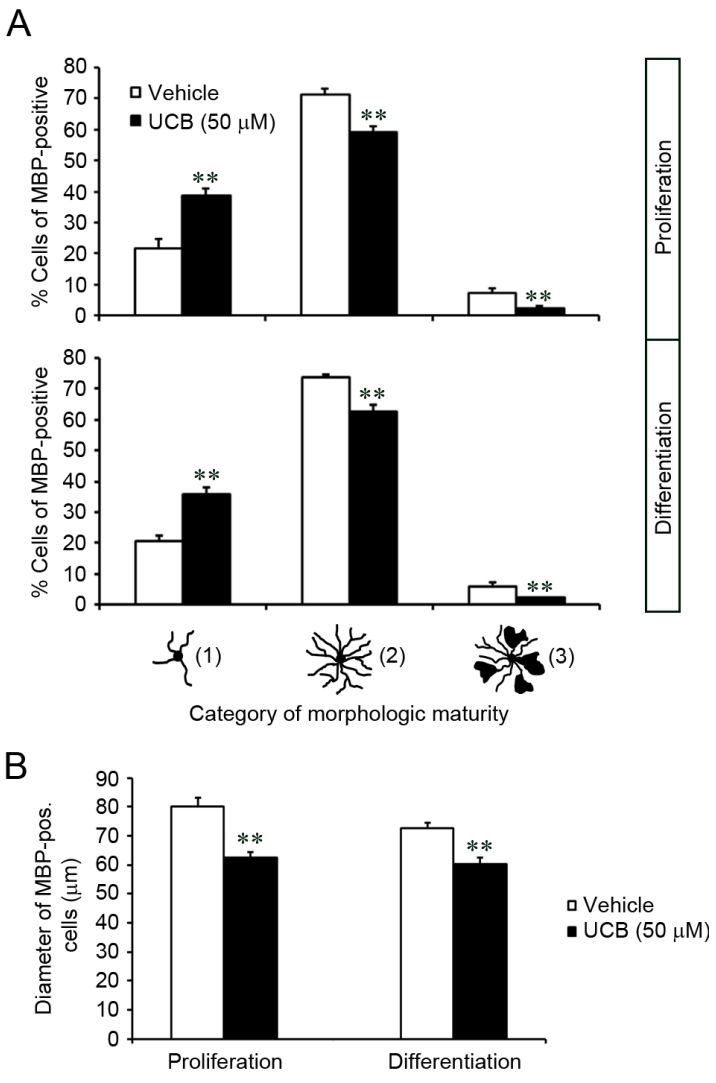


Fig. III.2 – Oligodendrocyte morphological maturation and mature oligodendrocyte diameter are modified by unconjugated bilirubin (UCB) treatment. Rat cortical oligodendrocytes were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during the 24 h of proliferation or during the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte maturation. After 7 days *in vitro*, cells were immunostained for myelin basic protein (MBP) and positive cells categorised according to their increasing morphologic maturity: cells with poorly branched processes (1), cells with complex branched processes (2) and cells with complex branched processes that partially form membranes (3). Quantification of oligodendrocyte in different stages of morphological maturation (**a**) and of oligodendrocyte diameter (**b**) from at least four independent experiments performed in duplicate. **P<0.01 vs. respective vehicle.

By Scholl analysis, we observed that both UCB experimental paradigms led to a decrease in mature OL process arborisation measured by a reduction of the sum of crossings and the average number of intersections per Sholl ring for MBP+ cells compared to vehicle-treated cells (from 12 to 60 μm radius for proliferation and from 4 to 56 μm radius for differentiation, $P<0.05$) (Figs. III.3a, b). The maximum distance of mature OL processes was also decreased in the presence of UCB for both treatments (0.79±0.01 fold and 0.82±0.01 fold for UCB exposure at proliferation and differentiation, respectively) demonstrating that differences in crossing number were due to both reduced process complexity and process length. Interestingly, the UCB effect on the number of crossings was more evident when UCB exposure occurred during initial differentiation, suggesting that UCB may also be acting on the cytoskeleton reorganization that occurs during process ramification, an essential event for the initiation of myelination.

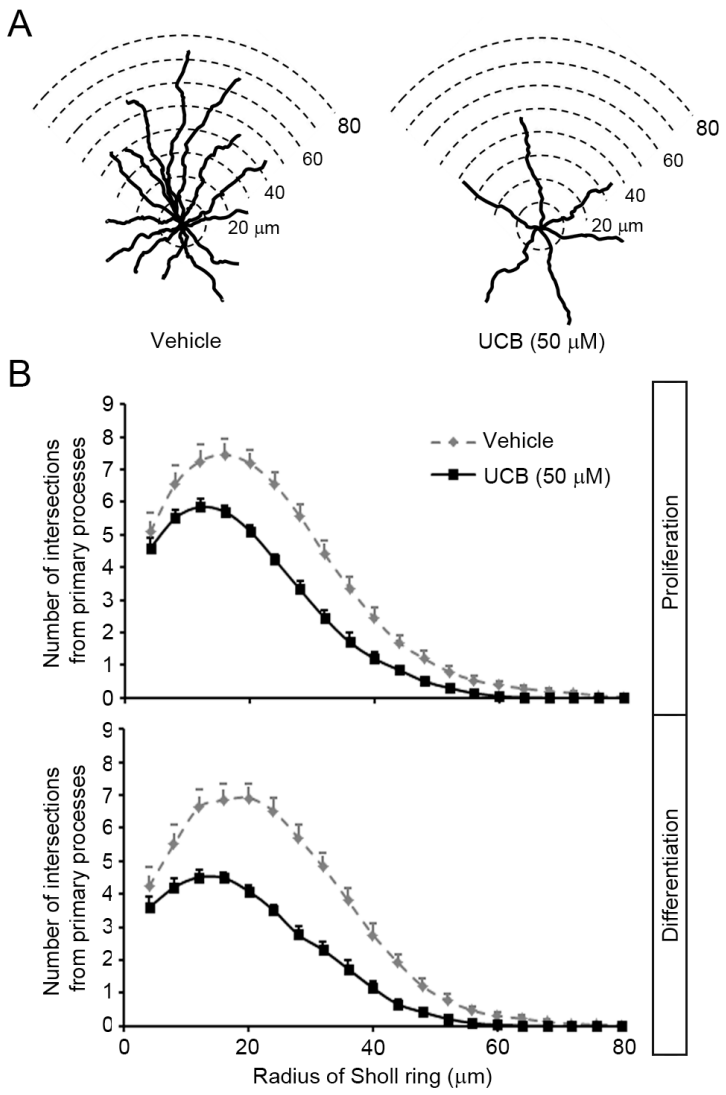


Fig. III.3 – Unconjugated bilirubin (UCB) reduces the primary branching density and elongation of mature oligodendrocytes. Rat cortical oligodendrocytes were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during the 24 h of proliferation or during the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte maturation for 7 days *in vitro*. **a** Representative tracings of the primary processes of a mature oligodendrocyte non-treated and treated with UCB. An overlay of concentric rings centred at the cell body was used for Sholl analysis. Concentric circles are spaced 4 μm apart. **b** Sholl analysis of primary processes measured at various concentric circles from at least 50 MBP+ cells in each condition.

Since, differential activation of small Rho GTPases, such as Rac1 and RhoA, has been described to impact OL differentiation (Liang et al. 2004), we next decided to evaluate if their activation was affected by UCB. These molecules, cycling between an active (GTP bound) and an inactive (guanosine diphosphate, GDP bound) state, influence OL shape by regulating actin upon activation from various stimuli (Feltri et al. 2008). In this context, we observed a marked decrease in active, GTP-bound Rac1 fraction after UCB treatment during proliferation and differentiation (0.15±0.06 fold and 0.42±0.03 fold, respectively, *P*<0.01) (Fig. III.4). Nevertheless, we did not detect the GTP-bound Rho fraction in either vehicle-treated or UCB-treated cells. These data suggest that UCB may impair actin cytoskeleton reorganization, by halting Rac1 activation, which is necessary for mature OL processes formation.

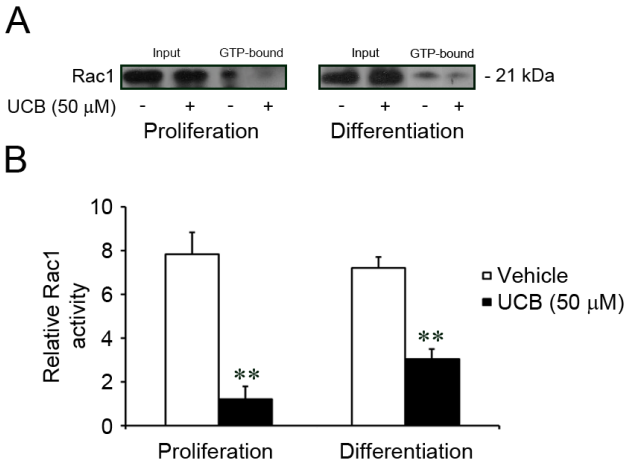


Fig. III.4 – Unconjugated bilirubin (UCB) reduces Rac1 activity. Rat cortical oligodendrocytes were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during the 24 h of proliferation or during the first 24 h of differentiation, and kept in differentiation medium to promote oligodendrocyte maturation for 7 days *in vitro*. **a** Representative results of immunoblotting from pull-down inputs and active guanosine triphosphate (GTP)-bound Rac1 fraction. **b** Decrease in relative Rac1 activity from vehicle (mean ± SEM) obtained for protein band intensity by scanning densitometry normalized to respective pull-down input from at least three independent experiments. ***P*<0.01 vs. respective vehicle.

3.3. UCB impairs differentiation of oligodendrocyte progenitors into myelinating cells in myelinating co-cultures

To access the effect of UCB on myelination, myelinating co-cultures of OL and DRG neurons were treated with UCB at 1 DCC (before the onset of myelination) and 7 DCC (during myelination), and cells were analysed at 15 DCC when myelination is complete. As shown in Fig. III.5, UCB exposure before myelination resulted in a decrease of MBP+ cells (0.69±0.04 fold, *P*<0.01) in parallel with an increase in NG2+ cells (1.42±0.07 fold, *P*<0.01). UCB exposure during myelination, following OPC differentiation, did not affect MBP+ cell numbers.

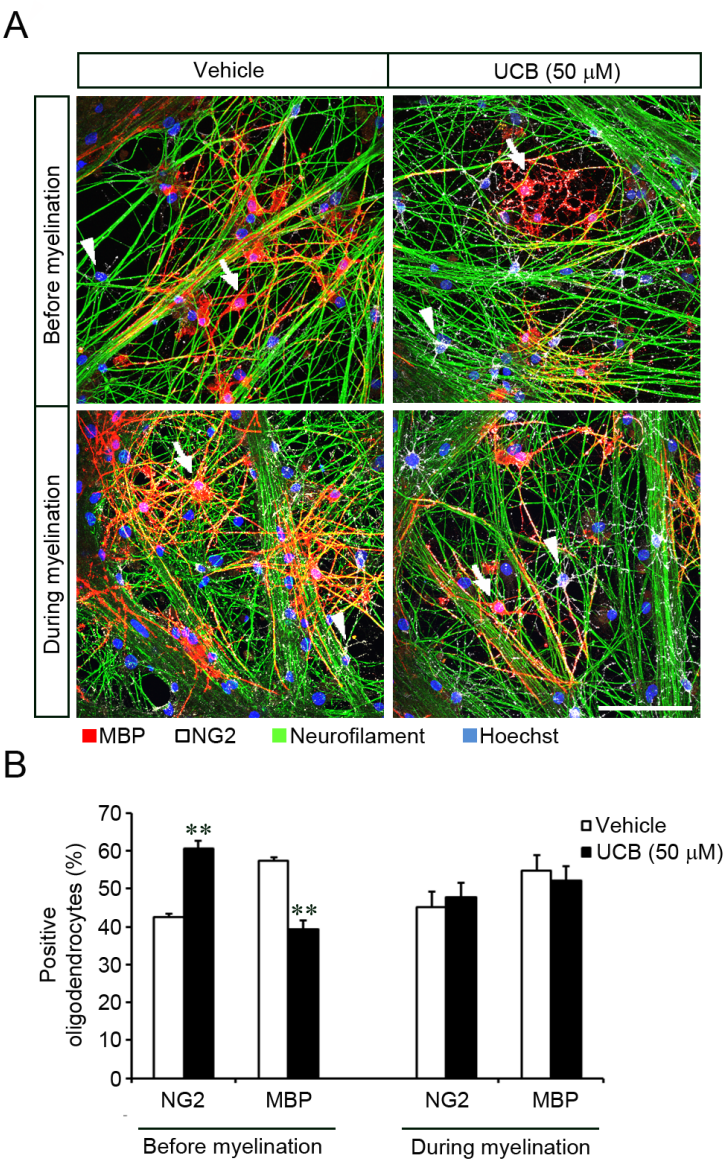


Fig. III.5 – Unconjugated bilirubin (UCB) impairs oligodendrocyte maturation in a co-culture myelinating model if exposure occurs before the beginning of myelination. Co-cultures of oligodendrocytes and dorsal root ganglia neurons were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during 24 h either at the beginning of myelination (1 day in co-culture, DCC) or during myelination (7 DCC), and allowed to recover until 15 DCC. The cells were immunolabelled to identify oligodendrocyte precursor cells (NG2, white, arrow heads), mature oligodendrocytes (myelin basic protein, MBP, red, arrow) and neuronal axons (neurofilaments, green). Nuclei were counterstained with Hoechst dye (blue). **a** Representative images show reduced myelination when UCB treatment occurred before the onset of myelination (top row) or during myelination (bottom row). **b** Quantification of the relative numbers of NG2+ and MBP+ cells following the different UCB treatments, from at least five independent experiments performed in duplicate. Scale bar represents 75 μm. ***P*<0.01 vs. respective vehicle.

3.4. UCB reduces oligodendrocyte process extension towards the axon and myelination

For myelination, mature OL extend their processes towards axons and wrap them to form the internode. Therefore, to evaluate the effect of UCB on mature OL morphological changes, we next categorised the MBP+ cells into three different stages based on process extension and axonal contact (Fig. III.6a). As shown in Fig. III.6b, exposure of the co-cultures to UCB prior to myelination led to a decrease in MBP+ cells wrapping axons (0.76±0.02 fold, *P*<0.01) or establishing contacts with axons (0.63±0.07 fold, *P*<0.01) while to a higher percentage of mature OL only extending processes without contacting axons (2.79±0.76 fold, *P*<0.01). Exposure to UCB during myelination also decreased percentage of mature OL wrapping axons (0.69±0.03 fold,

P<0.01) and increased those extending processes without contacting axons (2.95±0.45 fold, *P*<0.01). These results suggest that UCB exposure during myelination plays a role in the morphological alterations of the mature OL independent of effects on differentiation, thus impairing the ability of mature OL to myelinate.

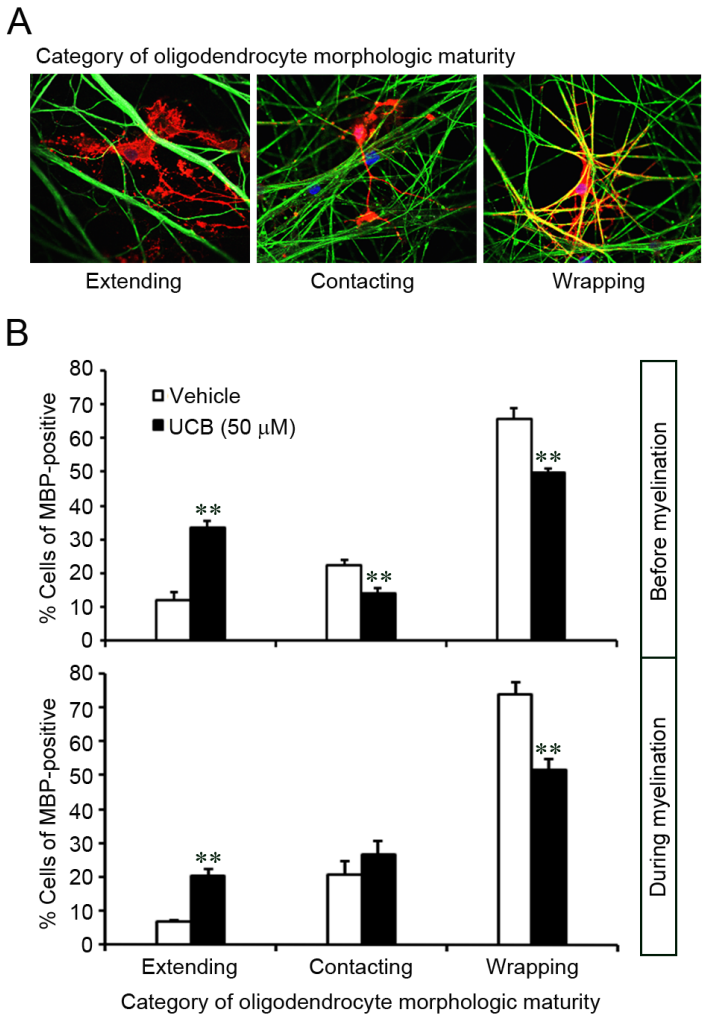


Fig. III.6 – In a myelinating co-culture model, unconjugated bilirubin (UCB) is able to induce a decrease in oligodendrocyte morphological maturation. Co-cultures of oligodendrocytes and dorsal root ganglia neurons were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during 24 h, either at the beginning of myelination (1 day of co-culture, DCC) or during myelination (7 DCC), and allowed to recover until 15 DCC. The cells were immunolabelled to identify mature oligodendrocytes (myelin basic protein, MBP, red) and neuronal axons (neurofilaments, green). Nuclei were counterstained with Hoechst dye (blue). **a** Representative images from different oligodendrocyte morphological maturation stages: cells that only extend processes but do not form contacts with axons, cells that establish contact with axons but do not myelinate, and cells that enwrap axons having at least two internodes. **b** Quantification of MBP+ cells by categorisation of oligodendrocyte morphologic maturity, from at least five independent experiments performed in duplicate. ***P*<0.01 vs. respective vehicle.

UCB reduced the number of internodes per mature OL if added either prior to (0.50±0.06 fold, *P*<0.01) or during myelination (0.71±0.02 fold, *P*<0.01) (Fig. III.7), and also reduced internodal length (0.58±0.03 fold or 0.77±0.02 fold for prior or during myelination, respectively, *P*<0.01).

Taken together, these results demonstrate that UCB exposure leads to myelination deficiency with more marked effects when UCB treatment occurs before the onset of myelination.

In order to verify if impaired myelination was due to axonal damage, we decided to determine the percentage of area occupied by neurofilaments. As shown in Suppl. Fig. III.1, we did not observe any significant changes in neurofilaments after UCB

exposure, indicating that myelination impairment is mainly due to alterations in OL development.

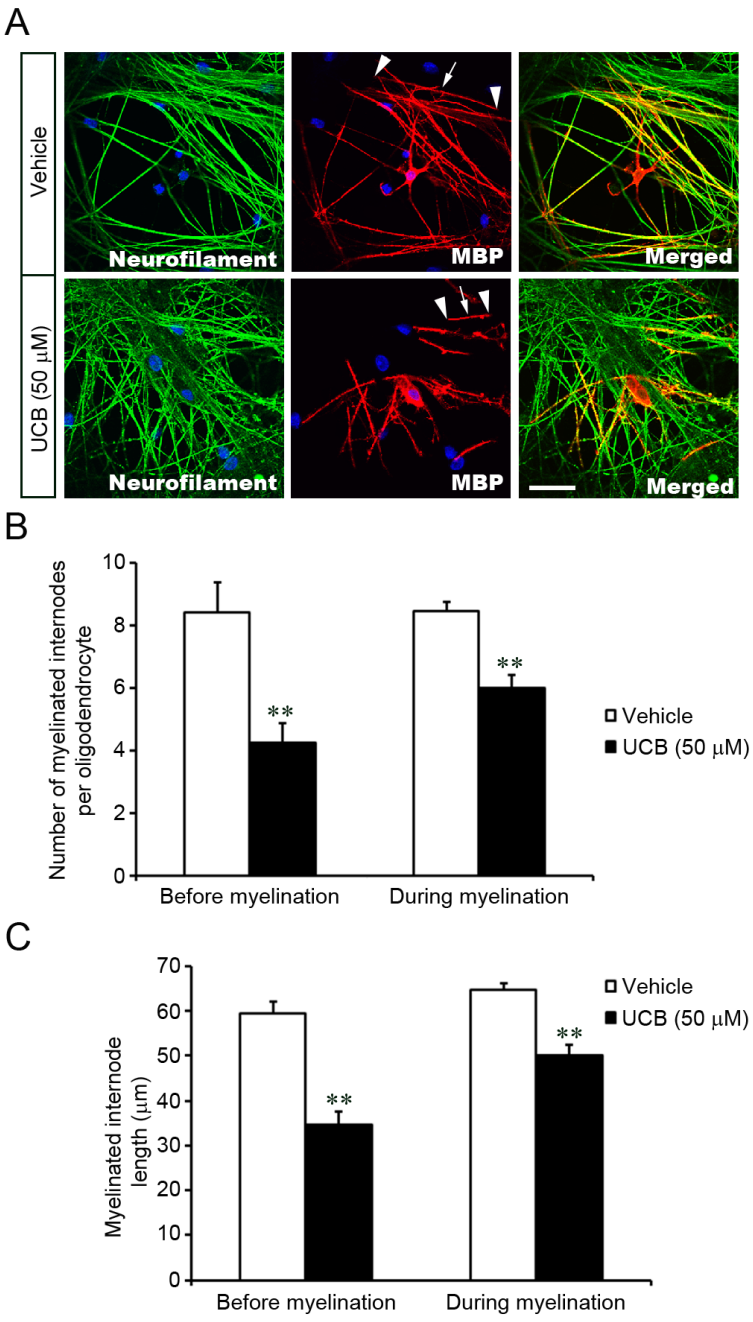


Fig. III.7 – Unconjugated bilirubin (UCB) treatment leads to a decrease in the number of internodes myelinated per each oligodendrocyte, as well as in the internode length. Co-cultures of oligodendrocytes and dorsal root ganglia neurons were exposed to 50 μM UCB in the presence of 100 μM human serum albumin during 24 h, either at the beginning of myelination (1 day of co-culture, DCC) or during myelination (7 DCC), and allowed to recover until 15 DCC. The cells were immunolabelled to identify mature oligodendrocytes (myelin basic protein, MBP, red) and neuronal axons (neurofilaments, green). Nuclei were counterstained with Hoechst dye (blue). **a** Representative images from non-treated (vehicle) and UCB-treated cultures indicating the internodes (arrow) and the internode length (distance between two arrow heads). Number of internodes myelinated per oligodendrocyte (**b**) and myelinated internode length (**c**) from at least five independent experiments performed in duplicate. ***P*<0.01 vs. respective vehicle.

4. Discussion

We report here that exposure of OL to UCB compromises myelinogenesis. Myelin abnormalities are often found after insults occurring during the neonatal period. Jaundice, sepsis and hypoxia are the most common clinical conditions in this period. Moreover, hypoxia/ischemia (Volpe 2001), chronic hypoxia (Vaccarino and Ment 2004),

and infection with inflammation involving pro-inflammatory cytokines (Dammann and Leviton 2004) are associated with white matter defects in infants.

Hypomyelination can be the result of either a reduced number or a dysfunction of mature OL. In addition, the number of mature OL depends on the prior proliferation and differentiation of OPC that are controlled by local growth/trophic factors. Our results show that UCB impairs the timing of OPC differentiation, with a significant effect if exposure to UCB occurs during OPC proliferation rather than during OPC initial differentiation. Indeed, when cells were analysed 7 DIV after inducing differentiation, UCB-treated cultures were in a developmentally immature state compared to vehicle-treated cells. These findings are in agreement with previous observations in chronic perinatal white matter injury showing a markedly expanded population of immature OL that display persistent maturation arrest with failure to differentiate (Segovia et al. 2008). As observed in our study, others have also reported a decrease in MBP expressing mature OL in differentiating cultures when treated with interferon (IFN)-γ alone or in combination with tumor necrosis factor (TNF)-α (Mann et al. 2008). Moreover, a recent study performed in animals treated with interleukin (IL)-1β also demonstrated an increase in OPC and immature OL and a decrease in premyelinating and myelinating OL (Favrais et al. 2011). Thus, it seems that UCB, like other pro-inflammatory molecules may prevent or impair OPC differentiation into myelinating mature OL.

Our previous results have shown that UCB reduces OPC survival through mitochondrial dysfunction and ER stress (Barateiro et al. 2012). Nevertheless, UCB treatment did not affect cell densities in the present study. Hence, the greater percentage of immature cells surviving after 7 DIV in differentiation medium could not be attributed to a lower cell density at the onset of differentiation. In accordance, other studies have already pointed out alterations in OL maturation and myelination without OL loss in periventricular leukomalacia (Billiards et al. 2008) or after cytokine treatment (Feldhaus et al. 2004).

The impairment observed in the maturation process due to UCB exposure can be in part explained by differences in Olig1 and Olig2 mRNA expression levels, since these transcription factors are crucial for OL development. In this context, while Olig2 is involved in specification to the OL lineage, Olig1 is involved in the final stages of myelin production (Copray et al. 2006; Lu et al. 2002). A recent study with human OL demonstrated that Olig1 expression peaks during membrane maintenance and that Olig2 is detected in freshly isolated cells, but absent during the process outgrowth and membrane maintenance (Othman et al. 2011). Moreover, Olig1 is essential for OL myelinogenesis in the brain and is required for transcription of MBP, proteolipid protein

(PLP) and myelin associated glycoprotein (MAG) (Xin et al. 2005). In accordance, we observed a significant decrease in Olig1 mRNA levels when UCB exposure occurred during the proliferation stage, which may partially justify the reduced OL maturation observed upon UCB treatment. We observed a concomitant increase in Olig2 mRNA expression following UCB exposure, which may be correlated to the increased number of NG2+ cells in UCB-treated cultures when compared to vehicle treatment. Interestingly, it has also been reported that a strong upregulation of Olig2, but not Olig1, occurs after acute brain injury, such as stab wound or focal ischemia, and in a model of chronic amyloid deposition (Buffo et al. 2005). Thus, we hypothesize that upon UCB exposure, mature OL reduce their ability to extend processes as a consequence of Olig1 downregulation, while the higher number of OPC present in UCB-treated cultures upregulate Olig2 to respond to UCB challenge.

Before myelination, premyelinating OL undergo a period of extensive process extension and retraction, and myelination itself is associated with morphological changes in the mature OL. In line with this, we found that OL maturation was greatly reduced by UCB treatment either during OPC proliferation or initial differentiation. In accordance with the observed reduction in MBP+ cells, UCB treatment impairs the extension of the processes and decreases the membrane surface of mature OL. This data is attributable to Rac1 not being allowed to transform from a GDP inactive state to a GTP active form due to the presence of UCB. Studies performed in cell cultures from postnatal rat brain have shown that inflammatory cytokines such as TNF-α and IFN-γ prevent the morphological differentiation of pre-myelinating OL (Agresti et al. 1996; Andrews et al. 1998; Mann et al. 2008), and changes in process extension were observed in a subset of periventricular leukomalacia cases (Billiards et al. 2008; Segovia et al. 2008). In a recent study, mitochondrial respiratory chain complex IV inhibition impaired process formation within OL lineage cells (Ziabreva et al. 2010). UCB also causes OPC mitochondria dysfunction (Barateiro et al. 2012), which may account for the reduced morphological maturation of mature OL we observed. Taken together, our data suggest that UCB does not affect mature OL survival, but rather affects the differentiation process, impairing the transition from OPC to mature OL.

Impaired process formation of OL (shorter and fewer processes) and failure to produce MBP expressing membranes following treatment with UCB may suggest a direct damage to cells by this biomolecule. It is known that differential activation of GTPases expressed by OL, like Rho and Rac1, is largely involved in cytoskeletal remodelling and in OL differentiation, and may play an important role in the morphological development of OL in response to UCB exposure. GTP-bound Rac1 build filamentous actin while GTP-bound Rho depolymerizes actin filamentous, having

a significant impact in cell morphology (Feltri et al. 2008). In our study, we were not able to detect the GTP-bound Rho fraction both in vehicle- or UCB-treated cells, but we observed a significant decrease in GTP-bound Rac1 fraction after UCB treatment during proliferation and differentiation. These results can explain the differences observed in process formation in UCB-treated cells. Indeed, others have reported that depletion of GTP-bound Rac1 in OL results in morphological defects (Wang et al. 2009). Our studies are also in line with a previous report demonstrating that inactivation of RhoA and activation of Rac1 increases OL differentiation (Liang et al. 2004). Interestingly, as demonstrated by others, Rac1 is important for myelin sheath formation (Czopka et al. 2009). We have previously shown that UCB impairs nerve cells cytoskeleton, with marked changes in neuronal neurofilaments and tubulin as well as in astrocyte glial fibrillary acidic protein (GFAP) and tubulin after 4 h treatment in conditions mimicking a severe hyperbilirubinemia (UCB/HSA=3) (Silva et al. 2002). In addition, early exposure to moderate levels of UCB (UCB/HSA=0.5) affects neuronal dendritic and axonal elongation as well as branching, with a marked reduction of growth cone area and filopodia (Fernandes et al. 2009).

Recent studies suggest that myelin plays an important role in learning and memory function (Fields 2008; Miki et al. 2009), likely due to its involvement in information processing and conduction velocity regulation. In the present study, reduced myelination in co-cultures can be explained by effects of UCB on OPC differentiation and pre-myelinating OL maturation. UCB added before the onset of myelination leads to decreased OPC differentiation with a consequent increase in OPC, whereas the same is not observed if incubation with UCB is performed during myelination. Thus, UCB impairs OPC differentiation only if present during OPC proliferation, and not if OPC are already committed to differentiate. Given the fact that OPC become postmitotic upon birth (Miller 2002), these data may justify why pre-term babies are more prone to myelination defects than term neonates. On the other hand, UCB incubations, either prior to or after the onset of myelination, affect premyelinating OL process elongation and axonal wrapping, as well as the number of myelinated internodes and the internode length. These results suggest that UCB may have an impact on premyelinating OL possibly at the cytoskeletal level, as observed in the OL differentiation cultures. In fact, myelin abnormalities with decreased MBP expression have already been observed in conditions that mimic typical insults during neonatal period like LPS-induced inflammation (Pang et al. 2003) or IFN-γ overexpression during CNS development (Lin et al. 2005). Postnatal sepsis and hypoxia-ischemia are also able to account for myelination impairment with a decrease in MBP expression (Huang et al. 2009; Loron et al. 2011). Induced moderate systemic inflammation during

the perinatal period has shown to alter the developmental programs of the white matter, causing long-term consequences in terms of susceptibility to adult brain diseases (Favrais et al. 2011). In this regard, neonatal hyperbilirubinemia has been likewise associated with an increased risk for the emergence of developmental delay, attention-deficit disorder and isolated neural hearing loss (Gkoltsiou et al. 2008), schizophrenia (Radhakrishnan et al. 2011) and autism (Amin et al. 2011). So these effects may be in part due to myelination changes induced by UCB during the first postnatal days.

Collectively, here we demonstrate for the first time that UCB may directly cause injuries to developing OL and impair myelination, justifying myelination defects observed in cases of severe neonatal jaundice (Brito et al. 2011; Gkoltsiou et al. 2008). Our results suggest that incomplete myelination following hyperbilirubinemia may derive from failure of OPC to differentiate and of premyelinating OL to complete the maturation and myelination programs. However, given our past results on neurons, we may also hypothesise that failure of OPC recruitment may result from imbalance in axonal guidance, or reduced attraction of axons to myelin sheath. In summary, once the myelin-forming process occurs in perinatal life, and continues through adolescence/early adulthood, alterations in developmental myelination by neonatal hyperbilirubinemia, as demonstrated *in vitro*, may have important implications for the pathogenesis of cognitive and/or psychiatric disorders associated with this common condition.

5. References

Agresti C, D'Urso D, Levi G. 1996. Reversible inhibitory effects of interferon-gamma and tumour necrosis factor-alpha on oligodendroglial lineage cell proliferation and differentiation in vitro. Eur J Neurosci 8(6):1106-16.

Ahdab-Barmada M, Moossy J. 1984. The neuropathology of kernicterus in the premature neonate: diagnostic problems. J Neuropathol Exp Neurol 43(1):45-56.

Amin SB, Smith T, Wang H. 2011. Is neonatal jaundice associated with Autism Spectrum Disorders: a systematic review. J Autism Dev Disord 41(11):1455-63.

Andrews T, Zhang P, Bhat NR. 1998. TNFalpha potentiates IFNgamma-induced cell death in oligodendrocyte progenitors. J Neurosci Res 54(5):574-83.

Barateiro A, Vaz AR, Silva SL, Fernandes A, Brites D. 2012. ER stress, mitochondrial dysfunction and calpain/JNK activation are involved in oligodendrocyte precursor cell death by unconjugated bilirubin. Neuromol Med [Epub ahead of print].

Barres BA, Lazar MA, Raff MC. 1994. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120(5):1097-108.

Billiards SS, Haynes RL, Folkerth RD, Borenstein NS, Trachtenberg FL, Rowitch DH, Ligon KL, Volpe JJ, Kinney HC. 2008. Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. Brain Pathol 18(2):153-63.

Bogler O, Wren D, Barnett SC, Land H, Noble M. 1990. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proc Natl Acad Sci U S A 87(16):6368-72.

Brites D. 2011. Bilirubin injury to neurons and glial cells: new players, novel targets, and newer insights. Semin Perinatol 35(3):114-20.

Brito MA, Zurolo E, Pereira P, Barroso C, Aronica E, Brites D. 2011. Cerebellar Axon/Myelin Loss, Angiogenic Sprouting, and Neuronal Increase of Vascular Endothelial Growth Factor in a Preterm Infant with Kernicterus. J Child Neurol.

Buffo A, Vosko MR, Erturk D, Hamann GF, Jucker M, Rowitch D, Gotz M. 2005. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. Proc Natl Acad Sci U S A 102(50):18183-8.

Bunge RP. 1968. Glial cells and the central myelin sheath. Physiol Rev 48(1):197-251.

Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E. 2006. Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. Stem Cells 24(4):1001-10.

Czopka T, Von Holst A, Schmidt G, Ffrench-Constant C, Faissner A. 2009. Tenascin C and tenascin R similarly prevent the formation of myelin membranes in a RhoA-dependent manner, but antagonistically regulate the expression of myelin basic protein via a separate pathway. Glia 57(16):1790-801.

Dammann O, Leviton A. 2004. Inflammatory brain damage in preterm newborns--dry numbers, wet lab, and causal inferences. Early Hum Dev 79(1):1-15.

Dewar D, Underhill SM, Goldberg MP. 2003. Oligodendrocytes and ischemic brain injury. J Cereb Blood Flow Metab 23(3):263-74.

Etienne-Manneville S, Hall A. 2002. Rho GTPases in cell biology. Nature 420(6916):629-35.

Favrais G, van de Looij Y, Fleiss B, Ramanantsoa N, Bonnin P, Stoltenburg-Didinger G, Lacaud A, Saliba E, Dammann O, Gallego J and others. 2011. Systemic inflammation disrupts the developmental program of white matter. Ann Neurol 70(4):550-65.

Feldhaus B, Dietzel ID, Heumann R, Berger R. 2004. Effects of interferon-gamma and tumor necrosis factor-alpha on survival and differentiation of oligodendrocyte progenitors. J Soc Gynecol Investig 11(2):89-96.

Feltri ML, Suter U, Relvas JB. 2008. The function of RhoGTPases in axon ensheathment and myelination. Glia 56(14):1508-17.

Fernandes A, Falcão AS, Abranches E, Bekman E, Henrique D, Lanier LM, Brites D. 2009. Bilirubin as a determinant for altered neurogenesis, neuritogenesis, and synaptogenesis. Dev Neurobiol 69(9):568-82.

Fields RD. 2008. White matter in learning, cognition and psychiatric disorders. Trends Neurosci 31(7):361-70.

Genc S, Genc K, Kumral A, Baskin H, Ozkan H. 2003. Bilirubin is cytotoxic to rat oligodendrocytes in vitro. Brain Res 985(2):135-41.

Gkoltsiou K, Tzoufi M, Counsell S, Rutherford M, Cowan F. 2008. Serial brain MRI and ultrasound findings: relation to gestational age, bilirubin level, neonatal neurologic status and neurodevelopmental outcome in infants at risk of kernicterus. Early Hum Dev 84(12):829-38.

Goldman JE. 1995. Lineage, migration, and fate determination of postnatal subventricular zone cells in the mammalian CNS. J Neurooncol 24(1):61-4.

Gurba PE, Zand R. 1974. Bilirubin binding to myelin basic protein, histones and its inhibition in vitro of cerebellar protein synthesis. Biochem Biophys Res Commun 58(4):1142-7.

Hansen T, Tommarello S, Allen J. 2001. Subcellular localization of bilirubin in rat brain after in vivo i.v. administration of [3H]bilirubin. Pediatr Res 49(2):203-7.

Hansen TW. 2000. Pioneers in the scientific study of neonatal jaundice and kernicterus. Pediatrics 106(2):E15.

Hansen TW. 2002. Mechanisms of bilirubin toxicity: clinical implications. Clin Perinatol 29(4):765-78, viii.

Huang Z, Liu J, Cheung PY, Chen C. 2009. Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury. Brain Res 1301:100-9.

Kitada M, Rowitch DH. 2006. Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in adult spinal cord. Glia 54(1):35-46.

Labombarda F, Gonzalez SL, Lima A, Roig P, Guennoun R, Schumacher M, de Nicola AF. 2009. Effects of progesterone on oligodendrocyte progenitors, oligodendrocyte transcription factors, and myelin proteins following spinal cord injury. Glia 57(8):884-97.

Lauer BJ, Spector ND. 2011. Hyperbilirubinemia in the newborn. Pediatr Rev 32(8):341-9.

Laursen LS, Chan CW, ffrench-Constant C. 2009. An integrin-contactin complex regulates CNS myelination by differential Fyn phosphorylation. J Neurosci 29(29):9174-85.

Liang X, Draghi NA, Resh MD. 2004. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. J Neurosci 24(32):7140-9.

Ligon KL, Fancy SP, Franklin RJ, Rowitch DH. 2006. Olig gene function in CNS development and disease. Glia 54(1):1-10.

Lin T, Xiang Z, Cui L, Stallcup W, Reeves SA. 2006. New mouse oligodendrocyte precursor (mOP) cells for studies on oligodendrocyte maturation and function. J Neurosci Methods 157(2):187-94.

Lin W, Harding HP, Ron D, Popko B. 2005. Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-gamma. J Cell Biol 169(4):603-12.

Loron G, Olivier P, See H, Le Sache N, Angulo L, Biran V, Brunelle N, Besson-Lescure B, Kitzis MD, Pansiot J and others. 2011. Ciprofloxacin prevents myelination delay in neonatal rats subjected to E. coli sepsis. Ann Neurol 69(2):341-51.

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 109(1):75-86.

Mann SA, Versmold B, Marx R, Stahlhofen S, Dietzel ID, Heumann R, Berger R. 2008. Corticosteroids reverse cytokine-induced block of survival and differentiation of oligodendrocyte progenitor cells from rats. J Neuroinflammation 5:39.

McCarthy KD, de Vellis J. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85(3):890-902.

McDonagh AF, Assisi F. 1972. The ready isomerization of bilirubin IX- in aqueous solution. Biochem J 129(3):797-800.

Miki K, Ishibashi S, Sun L, Xu H, Ohashi W, Kuroiwa T, Mizusawa H. 2009. Intensity of chronic cerebral hypoperfusion determines white/gray matter injury and cognitive/motor dysfunction in mice. J Neurosci Res 87(5):1270-81.

Miller RH. 1996. Oligodendrocyte origins. Trends Neurosci 19(3):92-6.

Miller RH. 2002. Regulation of oligodendrocyte development in the vertebrate CNS. Prog Neurobiol 67(6):451-67.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81(1):53-62.

Othman A, Frim DM, Polak P, Vujicic S, Arnason BG, Boullerne AI. 2011. Olig1 is expressed in human oligodendrocytes during maturation and regeneration. Glia 59(6):914-26.

Pang Y, Cai Z, Rhodes PG. 2003. Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. Brain Res Dev Brain Res 140(2):205-14.

Radhakrishnan R, Kanigere M, Menon J, Calvin S, Janish A, Srinivasan K. 2011. Association between unconjugated bilirubin and schizophrenia. Psychiatry Res 189(3):480-2.

Rubaltelli FF, Griffith PF. 1992. Management of neonatal hyperbilirubinaemia and prevention of kernicterus. Drugs 43(6):864-72.

Segovia KN, McClure M, Moravec M, Luo NL, Wan Y, Gong X, Riddle A, Craig A, Struve J, Sherman LS and others. 2008. Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. Ann Neurol 63(4):520-30.

Silberberg DH, Schutta HS. 1967. The effects of unconjugated bilirubin and related pigments on cultures of rat cerebellum. J Neuropathol Exp Neurol 26(4):572-83.

Silva RFM, Rodrigues CM, Brites D. 2002. Rat cultured neuronal and glial cells respond differently to toxicity of unconjugated bilirubin. Pediatr Res 51(4):535-41.

Syed YA, Baer AS, Lubec G, Hoeger H, Widhalm G, Kotter MR. 2008. Inhibition of oligodendrocyte precursor cell differentiation by myelin-associated proteins. Neurosurg Focus 24(3-4):E5.

Vaccarino FM, Ment LR. 2004. Injury and repair in developing brain. Arch Dis Child Fetal Neonatal Ed 89(3):F190-2.

Volpe JJ. 2001. Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res 50(5):553-62.

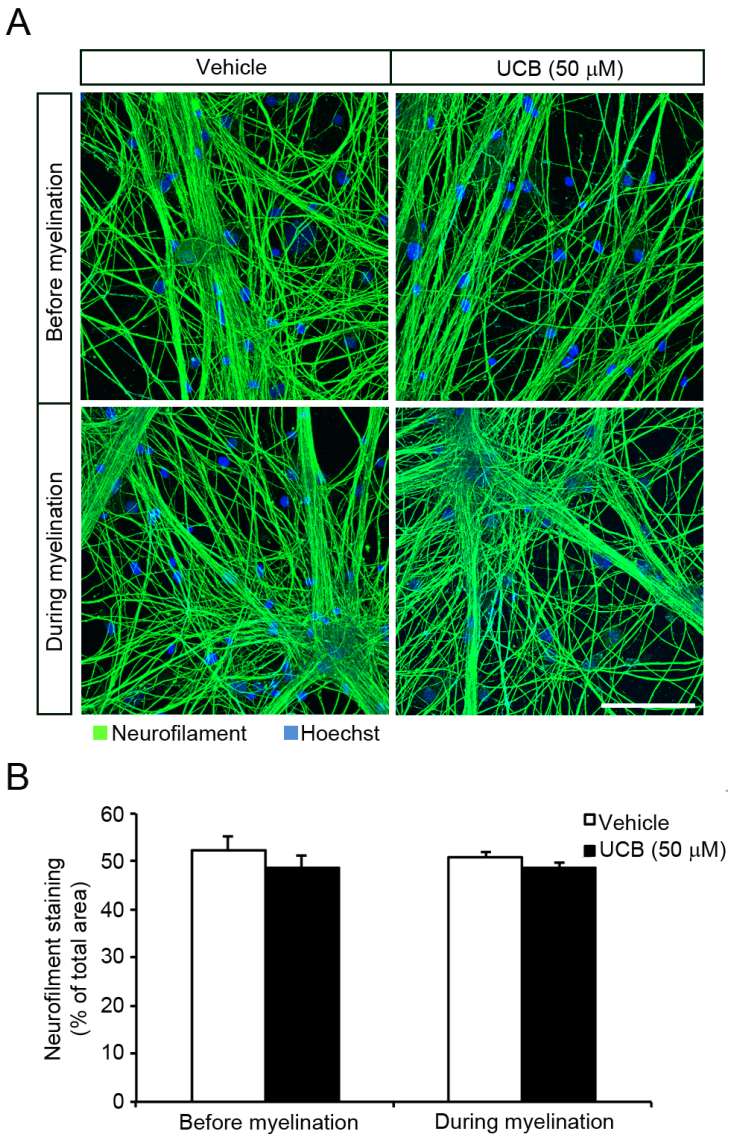
Wang PS, Wang J, Xiao ZC, Pallen CJ. 2009. Protein-tyrosine phosphatase alpha acts as an upstream regulator of Fyn signaling to promote oligodendrocyte differentiation and myelination. J Biol Chem 284(48):33692-702.

Xin M, Yue T, Ma Z, Wu FF, Gow A, Lu QR. 2005. Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. J Neurosci 25(6):1354-65.

Ziabreva I, Campbell G, Rist J, Zambonin J, Rorbach J, Wydro MM, Lassmann H, Franklin RJ, Mahad D. 2010. Injury and differentiation following inhibition of mitochondrial respiratory chain complex IV in rat oligodendrocytes. Glia 58(15):1827-37.

6. Supplementary Figures

6.1. Supplementary Figure III.1



Suppl. Fig. III.1 – Unconjugated bilirubin (UCB) did not induce axonal damage. Co-cultures of oligodendrocytes and dorsal root ganglia neurons were exposed to 50 µM UCB in the presence of 100 µM human serum albumin during 24 h, either at the beginning of myelination (1 day in co-culture, DCC) or during myelination (7 DCC), and allowed to recover until 15 DCC. The cells were immunolabelled to identify neuronal axons (neurofilaments, green). Nuclei were counterstained with Hoechst dye (blue). **a** Representative images showing identical neurofilaments immunoreactive area represented as percentage of total area. **b** Quantification of the area occupied by neurofilaments, expressed as percentage, following the different UCB treatments, from at least five independent experiments performed in duplicate. Scale bar represents 75 µm.

Chapter IV

CEREBELLUM SLICE CULTURE MODEL MIMICKING HYPERBILIRUBINEMIA: CHANGES IN OLIGODENDROCYTE MATURATION, MYELINOGENESIS AND GLIAL REACTIVITY

Andreia Barateiro[1], Helena S Domingues[2], Adelaide Fernandes[1,3], João B Relvas[2,4], Dora Brites[1,3]

[1]Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL),
Faculty of Pharmacy, University of Lisbon,
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal.

[2]Instituto de Biologia Molecular e Celular, University of Porto
Rua do Campo Alegre 823, 4150-180 Porto, Portugal

[3]Department of Biochemistry and Human Biology,
Faculty of Pharmacy, University of Lisbon
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

[4]Departamento de Biologia Experimental,
Faculty of Medicine, University of Porto
Av. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

PLOS One, 2012 (submitted)

Acknowledgments

This work was supported by the strategic project PEst-OE/SAU/UI4013/2011 and PTDC/SAU-NEU/64385/2006 grants from Fundação para a Ciência e a Tecnologia (FCT), Lisbon, Portugal (to D. B.). A. B. was recipient of a PhD fellowship (SFRH/BD/43885/2008) from FCT. H.S.D. is supported by a fellowship from European Commission (Marie Curie IEF 2010, #276322). Work in the lab of JBR was supported by a PTDC/BIA-BCM/112730/2009 from FCT. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Cerebellum is one of the brain regions most vulnerable to bilirubin-induced neurologic dysfunction. We recently demonstrated that unconjugated bilirubin (UCB) promotes oligodendrocyte progenitor cell (OPC) death and impairs oligodendrocyte maturation and myelination in co-cultures of dorsal root ganglia neurons and oligodendrocytes. Hence, we evaluated if dysregulation of myelination by UCB was also observed in the presence of other glial cells by using organotypic cerebellar slice culture as a model. Here, we demonstrated that UCB reduces the percentage of myelinated fibers and increases the OPC number in the cerebellar slice cultures. These UCB effects were associated with an enhanced gliosis, showing an increased burden of both microglia and astrocytes in the white matter tract. In addition, UCB treatment also led to a marked increase in tumor necrosis factor (TNF)-α and glutamate release, in parallel with a decrease in interleukin (IL)-6, and no changes in IL-1β and S100B secretion. Interestingly, all UCB effects were dependent on the time of exposure, presenting more marked alterations for longer periods. Taken together, these data point to a detrimental role of UCB in oligodendrocyte maturation, as well as in their ability to produce and support myelin, promoting gliosis together with the release of glutamate and TNF-α.

Keywords: Astrocytes, cerebellar slice culture, microglia, myelination, neuroinflammation, oligodendrocytes, unconjugated bilirubin.

1. Introduction

Neonatal jaundice is possibly the most common condition encountered by pediatricians due to an abnormal elevation of circulating unconjugated bilirubin (UCB) in the transitional period after birth. This syndrome can develop in newborns due to either excessive bilirubin production or impaired clearance, and the risk for developing bilirubin-induced neurologic dysfunction (BIND) depends on the degree of mismatch between bilirubin production and elimination (Cohen et al. 2010; Kaplan et al. 2002; Stevenson et al. 2001). The current understanding on BIND development is that when the level of serum UCB exceeds the bilirubin binding capacity of albumin, the extra unbound UCB readily crosses the blood-brain barrier (Bratlid 1990) and surpasses the protective mechanisms of the brain at preventing UCB accumulation (Brites 2012). In this situation it is known that UCB can cause apoptosis and necrosis, and the relative contribution of both types of cell death mechanisms depend on the severity and duration of damaging UCB cellular interaction, as well as on the type of cell (Brites and Brito 2012; Hanko et al. 2006a; Silva et al. 2002). In addition, glutamate is released from both neurons and astrocytes (Falcão et al. 2006; Fernandes et al. 2004; Stevenson et al. 2001) and treatment with the NMDA antagonist MK-801 protects neurons from UCB toxicity (Brito et al. 2010; Hanko et al. 2006b). Inflammation has also an important role in brain damage by UCB due to the immunostimulant effects on both astrocytes and microglia, leading to the release of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β (Fernandes et al. 2006; Fernandes et al. 2004; Gordo et al. 2006; Silva et al. 2011). In contrast, only a small secretion of TNF-α is produced when rat neuron primary cultures are treated with UCB, and no TNF-α and IL-1β secretion occurs when hippocampal slice cultures are used instead (Chang et al. 2009; Falcão et al. 2006). We and others have demonstrated that damage to developing neurons involves neuritic atrophy, reduced neuronal arborisation, arrested neuritic growth and neuritic hypoplasia (Fernandes et al. 2009; Shapiro 2005). Most important, our recent results demonstrated that UCB causes the death of oligodendrocyte precursor cells (OPC), through mitochondria dysfunction and endoplasmic reticulum stress (Barateiro et al. 2012a). Data also evidenced impaired oligodendrocyte (OL) maturation and deficient myelination in co-cultures with dorsal root ganglia (DRG) neurons exposed to UCB (Barateiro et al. 2012b).

Experimental *in vitro* models have been developed to study UCB toxicity and are a very common and useful methodology to understand the process of myelination. They are also important to isolate specific pathways and mechanisms of injury. However, since astrocytes and microglia are activated by UCB and are known to have

an important role in inflammation, synapse development and physiology (Castonguay et al. 2001; Lai and Todd 2008; Volterra and Meldolesi 2005), organotypic cultures made from slices of explanted tissue have the advantage of replicating many aspects of the *in vivo* system (Brites and Brito 2012). In fact, this model represent the complex multicellular environment, preserve cell relationships and extracellular matrix maintenance in a relatively intact three-dimensional structure, thus revealing to be a promising approach to understand the effects of UCB on the complexity of the myelination process. Moreover, besides avoiding several sources of artefacts and misinterpretations (Ghoumari et al. 2003; Kasparov et al. 2002; Schnadelbach et al. 2001) and allowing the open access to reproducible treatment with UCB, a recent study with mouse cerebellum slice cultures has demonstrated that both OPC and mature OL are present and compact myelin is formed (Zhang et al. 2011). Therefore, cerebellum slice cultures are a suitable model to evaluate the OL maturation process during which OPC experience dramatic morphological and biochemical changes (Craig et al. 2003). It is well established that OL undergo four distinct differentiation stages: OPC that express markers like NG2 proteoglycan, late progenitors that express the sulfatide O4, immature OL expressing galactosylceramidase and finally mature OL that express myelin proteins, such as myelin basic protein (MBP) (Buchet and Baron-Van Evercooren 2009). These myelin proteins are required not only for the saltatory conduction of neuronal action potentials, but also for maintenance of axonal integrity and fast axonal transport (Nave 2010).

In this study, we aimed to evaluate the effect of UCB in glial cell response, in OL development and in myelination, using the rat cerebellum slice culture model that allows the evaluation of myelination *in vitro*, while maintains the cell-cell interactions similar to those *in vivo*.

Our findings indicate that treatment of mouse cerebellum slice cultures with UCB leads to OL differentiation impairment, with the consequent increase in immature cells (NG2+ cells) and the decrease in the percentage of myelinated fibers. In addition, UCB has shown to enlarge the area occupied by astrocytes and microglia, enhancing the release of TNF-α and glutamate, as a result of UCB-induced reactivity of these cells. Interestingly, UCB effects reveal to be more pronounced for prolonged times of UCB exposure. UCB additionally causes a decrease in IL-6 release and do not change the production of either IL-1β or S100B.

2. Material and Methods

2.1. Reagents and media

Minimum essential medium Eagle (MEM), D-(+)-glucose solution 45% and bovine serum albumin fraction V (BSA) (fraction V, fatty acid free) were purchased from Sigma (St. Louis, MO, USA). UCB was also obtained from Sigma and purified according to the method of McDonagh (McDonagh and Assisi 1972). Normal horse serum (NHS), Hank's buffered salt solution (HBSS), 50 U/mL penicillin/50 µg/mL streptomycin and L-glutamine were acquired from Gibco (Life Technologies, Inc., Grand Islands, USA). Cell culture inserts for 6-well plates with 0.4 µm pores, translucent, high Density PET Membrane were from BD Falcon (#353493, Lincoln Park, NJ, USA). Rabbit antibody anti-NG2 was from Millipore (#AB5320, Billerica, MA, USA). Rat antibody anti-MBP was purchased from AbD Serotec (#MCA409S, Raleigh, NC, USA). Mouse antibody anti-neurofilament-medium [NF-09] was from Abcam (#ab7794, Cambridge, UK). Rabbit anti-glial fibrillary acidic protein (GFAP) (#G9269) and monoclonal anti-S100B capture antibody (#S2532) were from Sigma. Rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) was purchased from Wako (#019-19741, Osaka, Japan). Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rat IgC, Alexa Fluor 488 goat anti-rabbit IgG were acquired from Invitrogen Life Sciences (Carlsbad, CA, USA). Policlonal anti-S100B detection antibody was from Dako (#Z0311, Glostrup, Dennmark). Direct-Zol™ RNA MiniPrep was obtained from Zymo Research (Irvine, CA, USA). RevertAid H Minus First Strand cDNA synthesis and Maxima SYBR Green qPCR Master Mix (2x) were acquired from Fermentas (Ontario, Canada, USA). Recombinant rat TNF-α, IL-1β and IL-6 and DuoSet® ELISA kits were from R&D Systems, Inc. (Minneapolis, MN, USA). L-glutamic acid kit was from Roche Molecular Biochemicals (Mannheim, Germany). Shandon Immu-Mount™ Aqueous Non-fluorescing Mounting Medium was acquired from Thermo Scientific (Waltham, MA, USA).

2.2. Animals

Wistar rats were maintained on a 12 h light/dark cycle under conditions of constant temperature and humidity. Animals were supplied with standard laboratory chow and water ad libitum. Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the

Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

2.3. Cerebellar slice culture and treatment

Parasagittal slices were prepared from post-natal day (P) 7 Wistar rat cerebellum, according to the interface culture method. Briefly, brains were removed, cerebellum was isolated and 400 μm slices were made using a McIlwain tissue chopper. The slices were then placed in the upper chamber of a 0.4 μm pore cell culture insert and maintained in tissue culture plates containing 1 mL of medium in the plate well at 37ºC in room air and with 5% CO_2 for 7 days *in vitro* (DIV). The culture medium consisted of 50% MEM, 25% NHS, 6.5 mg/mL D-glucose and 0.5% penicillin/streptomycin. Half of the medium was replaced every day. Rat P7 cerebellar slices were not completely myelinated when placed in culture. During the time in culture, OL differentiation occurred until they filled the white matter of folia and started myelination. After 7 DIV, the cerebellar slice culture evidenced a high degree of myelination, based on the significant acquirement of the number of myelin processes aligned with axons.

Since we wanted to evaluate the effect of UCB in the *de novo* myelination, slice cultures were kept in culture for 3 DIV, to allow the clearance of debris and the stabilization of cerebellar slices, and then incubated with UCB prior to the emergence of compact myelin. Exposure of slices to the periods of 8 and 24 h interaction with UCB aimed to mimic a more acute (first) or chronic (second model) conditions, respectively.

High concentration of NHS is indispensable for the maintenance of cerebellar slice cultures and this serum contains an elevated concentration of albumin. So, in order to maintain the usual molar ratio of UCB/albumin of 0.5, we recalculated the concentration of UCB to achieve the 29 nM of free UCB, that corresponds to the model that we have been using in the last years and that showed to produce injurious effects of UCB on oligodendrogenesis and myelinogenesis (Barateiro et al. 2012b). Therefore, instead of using 50 μM UCB in the presence of 100 μM HSA as indicated in our previous reports, cultures were here treated with 60 μM UCB in the presence of 25% of NHS. All UCB solutions were prepared from a 10 mM stock solution in 0.1 N NaOH immediately before use and the pH of the incubation media was restored to 7.4 by addition of equal amounts of 0.1 N HCl. All the experiments with UCB were performed under light protection to avoid photodegradation. After incubation, the UCB containing medium was removed and replaced with fresh culture medium.

2.4. Immunostaining procedure

After 7 DIV, cerebellar slice cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at room temperature. Membranes containing tissue sections were cut out from cell culture insert and incubated with blocking solution [1 nM 4-(2-hidroxyethyl)-1-piperazineethanosulfonic (HEPES), 2% heat-inactivated horse serum, 10% heat-inactivated goat serum, 1% BSA and 0.25% Triton X-100 in HBSS] for 3 h to block nonspecific binding. The sections were incubated with primary antibodies diluted in blocking solution for 24 h at 4ºC. The following antibodies were used: neurofilament-medium (NF-m) for neuronal axons (1:50), NG2 for OPC (1:100), MBP for mature OL (1:50), GFAP for astrocytes (1:250) and Iba-1 for microglia (1:250). The next day, slices were washed three times for 30 min each with PBS before incubation for another 24 h at 4ºC with secondary antibody in blocking solution (1:1,000). Slices were then washed three times for 30 min and mounted for confocal microscopy. Percentage of the area immunoreactive for each antibody was measured in 40x magnification images acquired in a Laser Scanning Confocal Microscope Leica SP2 AOBS SE (Leica Microsystems, Germany). Briefly, binary masks were defined using the same cut-off intensity threshold value for each region of interest, which corresponds to each cell immunostained, defined as the minimum intensity due to specific staining above background values. Then, the percentage of the area occupied by neurofilaments, MBP, GFAP and Iba-1 were measured automatically using ImageJ software in each cerebellum region. Results are given by averaging values determined in at least seven separate microscopic fields from four slices of different animals. Values are expressed as mean ± SEM.

2.5. Semi-quantitative RT-PCR

Total RNA was extracted from each tissue section after 7 DIV using Direct-Zol™ RNA MiniPrep kit, according to the manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 1 µg of total RNA were treated with DNase I and then reverse transcribed into cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase under the recommended conditions. Quantitative RT-PCR (qRT-PCR) was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control to normalize the expression level of Olig1 and Olig2 transcription factors. The following sequences were used as primers: Olig1 sense 5'-GCCCAGGCCACGAGTACAAA-3' and anti-sense 5'-TCCACTCCGAAACCCAACGA-3' (Labombarda et al. 2009); Olig2 sense 5'-GAAATGGAATAATCCCGAACTACT-3' and

anti-sense 5'-CCCCTCCCAAATAACTCAAAC-3' (Labombarda et al. 2009); GAPDH sense 5'-TGGAGTCTACTGGCGTCTT-3' and anti-sense 5'-TGTCATATTTCTCGTGGTTCA-3'. qRT-PCR was performed on a real-time PCR detection (Applied Biosystems 7300 Fast Real-time PCR System, Applied Biosystem, Madrid, Spain) using a SYBR Green qPCR Master Mix. The PCR was performed in 96 well plates with each sample performed in triplicate, and a no-template control was included for each amplificate. qRT-PCR was performed under optimized conditions: 94ºC for 3 min followed by 40 cycles at 94ºC for 9 s, 60ºC for 12 s and 72ºC for 9 s. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not detected in any case. Relative mRNA concentrations were calculated using the Pfaffl modification of the $\Delta\Delta C_T$ equation (C_T, cycle number at which fluorescence passes the threshold level of detection), taking into account the efficiencies of individual genes. The results were normalized to GAPDH in the same sample and the initial amount of the template of each trial was determined as relative expression by the formula $2^{-\Delta\Delta CT}$. ΔC_T is the value obtained, for each sample, by performing the difference between the mean C_T value of each Olig gene and the mean C_T value of GAPDH. $\Delta\Delta C_T$ of one sample is the difference between its ΔC_T value and the ΔC_T of the sample chosen as reference, in our case the vehicle-treated cells.

2.6. Cytokine determination

Cytokine release by cerebellar slice cultures was quantified along the time-course of the experiments and following UCB exposure. Aliquots of the slice culture supernatants were placed in a 96-well microplate and assessed, in duplicate, for TNF-α, IL-1β and IL-6 with specific DuoSetR ELISA Development kits, according to the manufacturer's instructions. Measurements were performed at 450 nm, with a reference filter at 620 nm, using a microplate reader. Results are expressed as pg/mL.

2.7. S100B determination

S100B release to the culture media were determined by ELISA, as previously described (Leite et al. 2008). Briefly, supernatants were incubated for 2 h at 37ºC on a 96-well plate previously coated with a monoclonal anti-S100B antibody (1:1,000). Thereafter, a polyclonal anti-S100B antibody (1:5,000) was added and samples additionally incubated for 30 min at 37ºC. Finally, an anti-rabbit peroxidase-conjugated antibody (1:5,000) was added for further 30 min at 37ºC. The colorimetric reaction with

Sigma Fast OPD tablets was measured at 492 nm, using a microplate reader. Results are expressed in ng/mL.

2.8. Glutamate determination

Release of glutamate to the culture medium was determined by an adaptation of the L-glutamic acid kit (Roche), using a 10-fold volume reduction. The reaction was performed in a 96-well microplate and the absorbance measured at 490 nm. A calibration curve was used for each assay. All samples and standards were analysed in duplicate. Results are expressed in µM.

2.9. Statistical analysis

Results of, at least, three different experiments are expressed as mean ± SEM. Significant differences between groups were determined by the two-tailed *t*-test performed on the basis of equal and unequal variance and considering a minimal criterion of $P<0.05$ as statistically significant.

3. Results

3.1. UCB treatment impairs oligodendrocyte maturation and myelination

Here we tested the effect of UCB in cerebellar slice cultures. This *in vitro* culture system allows the study of OL-neuron relationship in a preserved brain cytoarchitecture that closely resembles the *in vivo* situation and, therefore, is commonly used to study myelination. For that, slice cultures were treated, at 3 DIV, with UCB during 8 h and 24 h to assay whether the extension of UCB exposure may differently affect OL maturation and myelination, as we have previously observed in other models namely in OPC treated with UCB (Barateiro et al. 2012a,b). Cerebellar slice cultures were maintained in culture for 7 DIV and then immunostained for NG2, neurofilaments and MBP, in order to determine the number of OL in a more immature stage and the percentage of myelinated fibers, respectively. As depicted in Fig. IV.1, both conditions of UCB treatment led to an increase in NG2+ cells, more elevated by the more prolonged exposure (2.26±0.58 fold at 8 h and 2.62±0.66 fold at 24 h, $P<0.01$).

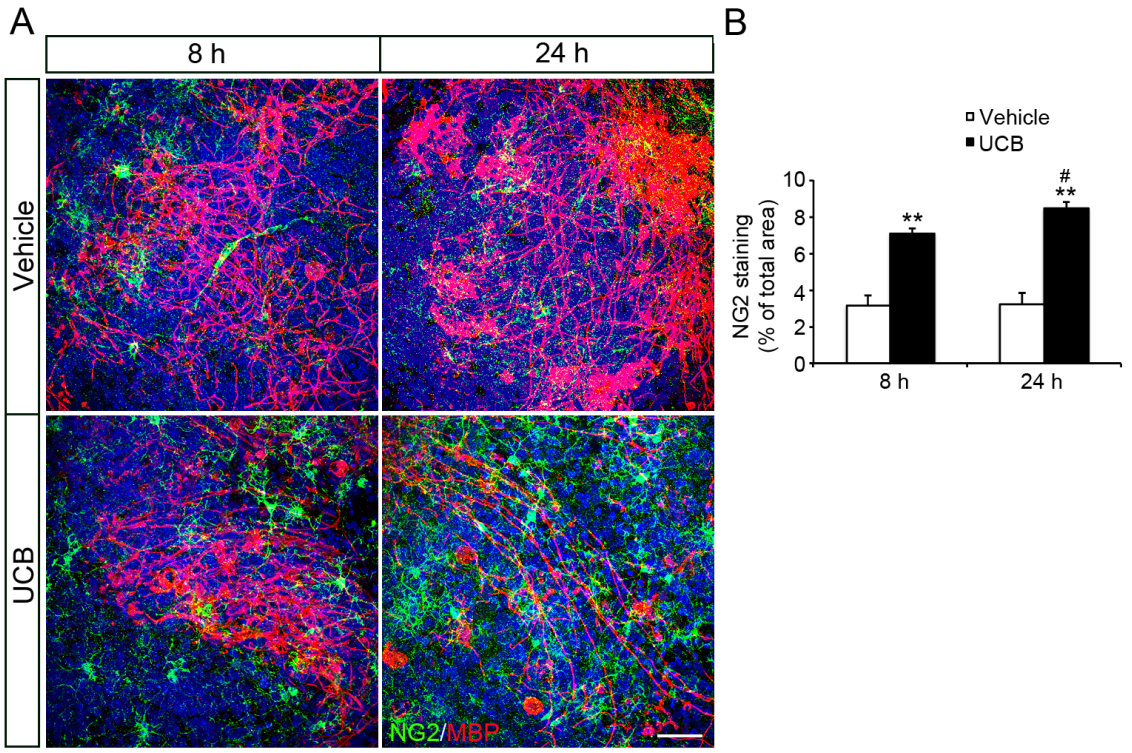


Fig. IV.1 – Impairment of oligodendrocyte (OL) differentiation by unconjugated bilirubin (UCB), in a rat cerebellar slice culture, increase with the time of exposure. Cerebellar slice cultures were exposed for 8 h and 24 h to UCB, at 3 day *in vitro* (DIV), and maintained in culture allowing the recover until 7 DIV. **a** The cells were immunolabelled to identify oligodendrocyte precursor cells (OPC, NG2+ cells, green) and mature OL (myelin basic protein positive cells, MBP, red). Nuclei were counterstained with DAPI dye (blue). Representative images show an increase in the area occupied by NG2+ cells after 8 h (left column) and 24 h (right column) with UCB. **b** Percentage of area occupied by NG2+ staining following treatments, from at least four independent experiments. Scale bar represents 50 µm. ***P*<0.01 vs. respective vehicle; $^{\#}P$<0.05 vs. 8 h of UCB exposure.

Regarding OL ability to myelinate axons (Fig. IV.2), the treatment with UCB has shown to induce a time-dependent decrease in the percentage of myelinated fibers (0.70±0.02 fold at 8 h and 0.48±0.02 fold at 24 h, *P*<0.01). Corroborating the immunohistochemical observations, we have also detected a very similar pattern in the decrease of MBP mRNA expression for both treatments (Fig. IV.2c), with a slightly higher reduction at 24 h as compared to only 8 h treatment (0.57±0.06 fold for 8 h and 0.48±0.05 fold for 24 h, *P*<0.01). These data suggest that presence of UCB triggers the delay in oligodendrocyte maturation, with an increase in OPC and a decrease in myelinated fibers. The time of exposure revealed to increase the magnitude of the effects produced.

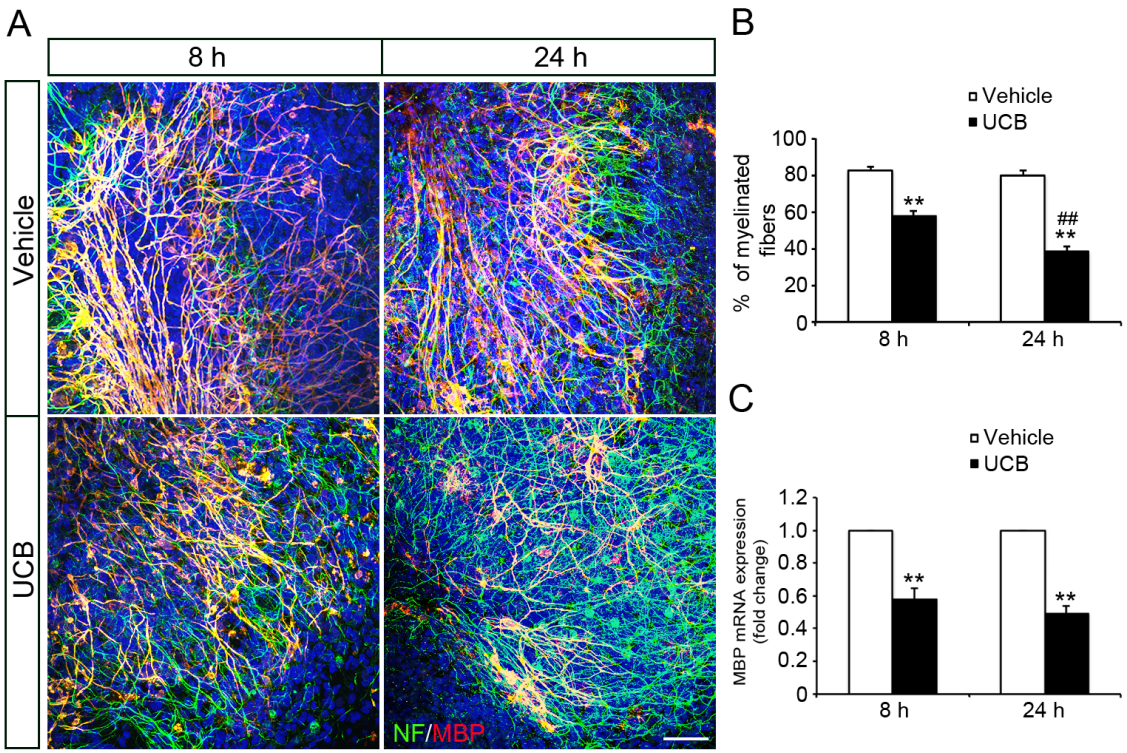


Fig. IV.2 – Impaired myelination by unconjugated bilirubin (UCB) in rat cerebellar slice cultures increases with the time of exposure. Cerebellar slice cultures were exposed to UCB at 3 day *in vitro* (DIV) for 8 h and 24 h, and allowed to recover until 7 DIV thereafter. **a** Oligodendrocytes were immunolabelled for myelin basic protein (MBP, red) and neuronal axons (neurofilaments, NF, green). Nuclei were counterstained with DAPI dye (blue). Representative images show reduced myelination after 8 h incubation with UCB (left column) that further decreased at 24 h (right column). **b** Percentage of myelinated fibers after both treatment periods, from at least four independent experiments. **c** Relative MBP mRNA levels determined by qRT-PCR with the $\Delta\Delta C_T$ method. Values are shown as mean ± SEM from at least five independent experiments performed in triplicate, normalized to values measured for glyceraldehyde 3-phosphate dehydrogenase in the same samples. Scale bar represents 50 μm. $^{**}P<0.01$ vs. respective vehicle, $^{\#\#}P<0.01$ vs. 8 h of UCB exposure.

3.2. Differences in Olig1 and Olig2 mRNA expression are invoved in oligodendrocyte maturation impairment by UCB

Since Olig1 and Olig2 are transcription factors that play essential roles in the differentiation of OL lineage cells, from glial precursor cells to fully mature myelinating OL (Dugas et al. 2006; Lu et al. 2000; Nicolay et al. 2007; Zhou and Anderson 2002), we decided to evaluate whether UCB treatment affected Olig1 and Olig2 mRNA expression during myelination in cerebellar slice cultures. Indeed, we have recently demonstrated that UCB reduces Olig1 mRNA and increases Olig2 mRNA in OL maturation, by using an *in vitro* model (Barateiro et al. 2012b). Similar results were now obtained by UCB treatment of cerebellar slices, as depicted in Fig. IV.3. In fact, once again we verified that UCB decreased Olig1 (0.76±0.05 fold for 8 h and 0.64±0.05 fold

for 24 h, *P*<0.01) and increased Olig2 mRNA expression (1.15±0.05 fold for 8 h and 1.58±0.15 fold for 24 h, *P*<0.01). Although both incubation periods with UCB have produced changes in gene expression, stronger effects have resulted with the longer time period treatment.

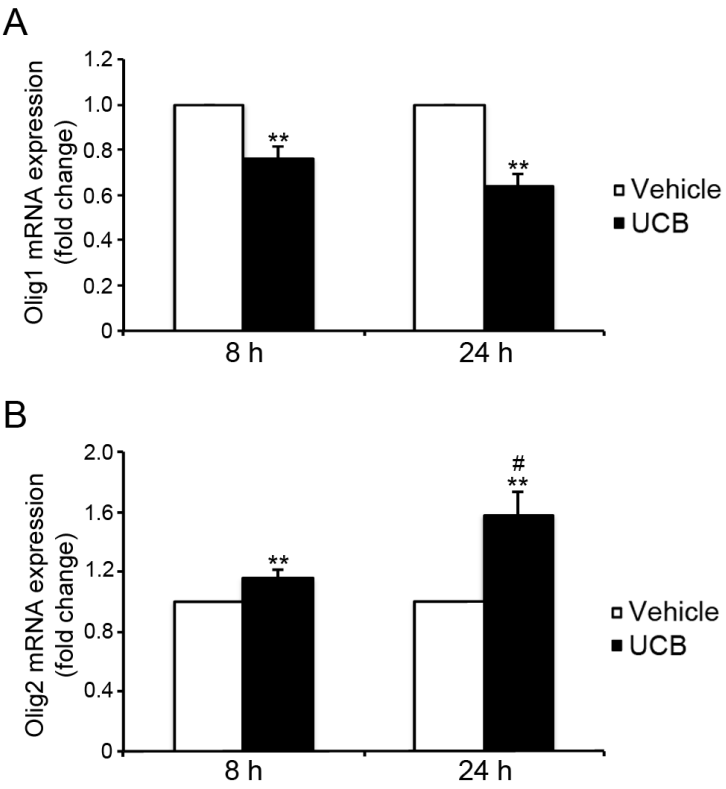


Fig. IV.3 – Unconjugated bilirubin (UCB) induces a reduction and an increase of Olig1 and Olig2 mRNA expression, respectively. Cerebellar slice cultures were exposed to UCB at 3 day *in vitro* (DIV) for 8 h and 24 h followed by maintenance in culture until 7 DIV. Relative levels of transcription factors Olig1 (**a**) and Olig2 (**b**) were determined by qRT-PCR with the $\Delta\Delta C_T$ method. Values are shown as mean ± SEM from at least five independent experiments performed in triplicate, normalized to values of glyceraldehyde 3-phosphate dehydrogenase in the same samples. ***P*<0.01 vs. vehicle, $^{\#}$*P*<0.05 vs. 8 h of UCB exposure.

3.3. UCB treatment stimulates astrogliosis and microgliosis

The presence of other glial cells, namely astrocytes and microglia, may influence the formation of the myelin sheath (Emery 2010). Having observed that UCB impairs myelinogenesis and based on our prior results demonstrating that UCB promotes both astroglial and microglial activation (Brites 2011), we wondered whether in this more similar *in vivo* model UCB could also affect OL in the presence of glial cells environment. Immunostaining of cerebellar slice cultures with specific antibodies for microglia at 7 DIV revealed an increased area percentage of Iba-1+ cells following UCB treatment, and even more when the exposure to UCB were prolonged from 8 to 24 h (1.59±0.15 fold and 1.74±0.24 fold, *P*<0.01, respectively) (Figs. IV.4a, b). Immunostaining for GFAP revealed the same tendency (1.33±0.15 fold for 8 h, *P*<0.01 and 1.60±0.42 fold for 24 h, *P*<0.01) (Figs. IV.4c, d). These results clearly show that besides damage to OL UCB also stimulates microgliosis and astrocytes in a time-dependent manner.

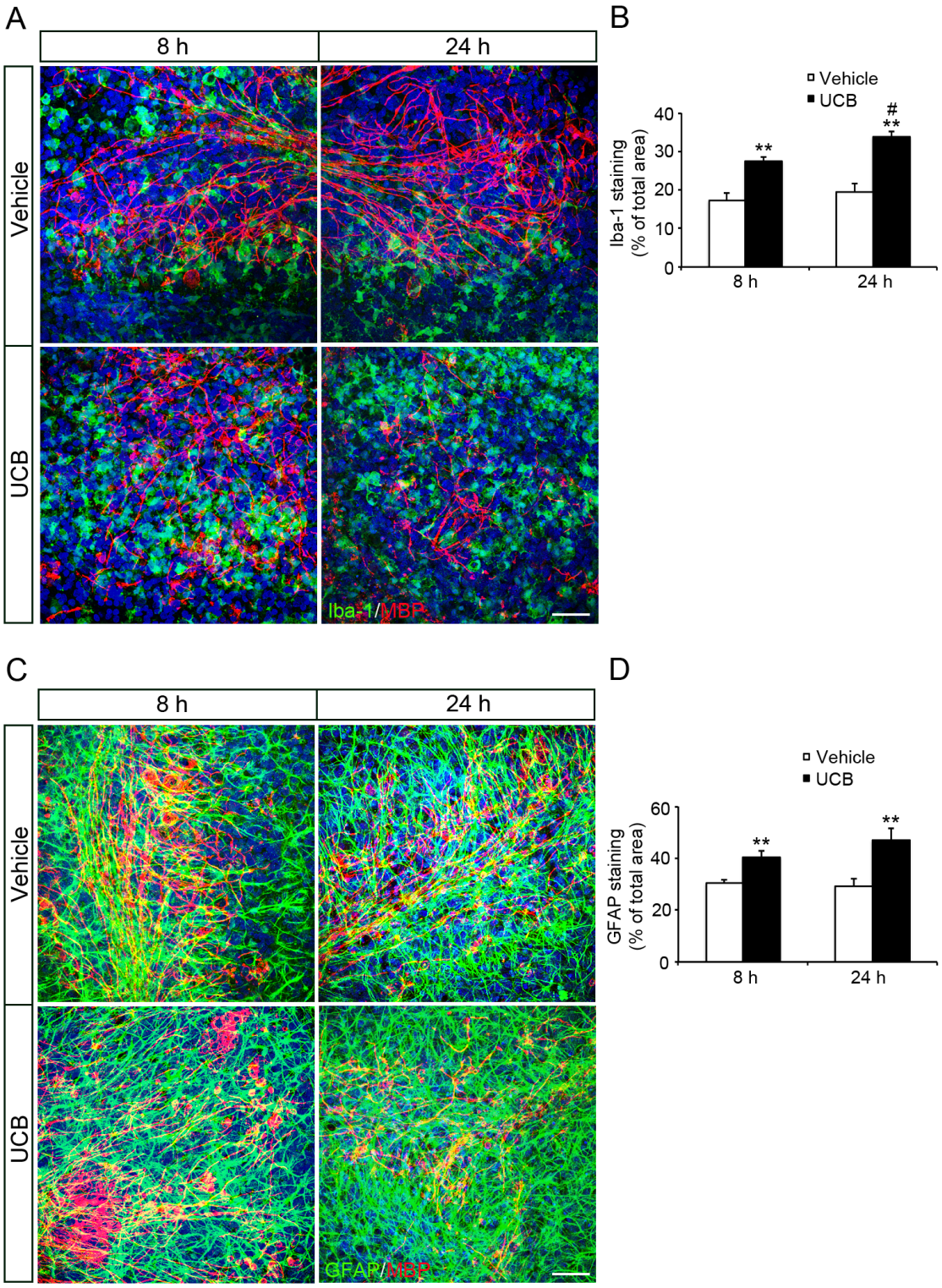


Fig. IV.4 – Unconjugated bilirubin (UCB) induces an increase in the number of microglia and astrocytes. Cerebellar slice cultures were exposed to UCB at 3 day *in vitro* (DIV) for 8 h and 24 h, and maintained in culture until 7 DIV. **a** Oligodendrocytes were immunolabelled for myelin basic protein (MBP, red) and microglia for Iba-1 (green). Nuclei were counterstained with DAPI dye (blue). Representative images show an increase in microglia number after UCB treatment for 8 h (left column) and 24 h (right column). **b** Quantification of the percentage of area occupied by Iba-1+ stained cells after both time periods, from at least four independent experiments. **c** Oligodendrocytes were immunolabelled to identify myelin basic protein (MBP, red) and astrocytes for GFAP (green). Nuclei were counterstained with DAPI dye (blue). Representative images show an increase in area occupied by GFAP+ cells after 8 h (left column) and 24 h (right column) of UCB exposure. **d** Quantification of the percentage of area occupied by GFAP+ staining at both time periods, from at least four independent experiments. Scale bar represents 50 µm. $^{**}P<0.01$ vs. respective vehicle, $^{\#}P<0.05$ vs. 8 h of UCB exposure.

3.4. UCB treatment induces the secretion of TNF-α while inhibits the release of IL-6

Supporting evidence indicates that UCB stimulates the production of pro-inflammatory cytokines by both astrocytes and microglia (Fernandes et al. 2004; Gordo et al. 2006). Therefore, we next decided to characterize the temporal secretion of TNF-α, IL-1β, IL-6 and S100B into the incubation media. The cellular release of these cytokines was determined before UCB treatment, after UCB exposure for 8 h or 24 h and in the following days until the culture reached the 7 DIV. As depicted in Fig. IV.5, cytokine release presents changes throughout the maintenance of the cerebellar slice culture until 7 DIV even in vehicle-treated cultures. This is due to the basal production of cytokines along the maintenance of slice cultures for the 7 DIV and its accumulation since only half of the medium was replaced. After 8 h of incubation, UCB clearly showed to produce a first elevation in the TNF-α production immediately after treatment (1.96±0.25 fold, $P<0.01$) that was maintained in the next 24 h (1.91±0.16 fold, $P<0.01$) and although slightly decreased at 5 DIV, increased again at days 6 and 7 DIV (2.55±0.96 fold and 1.59±0.13 fold, respectively, $P<0.01$). As expected, 24 h exposure to UCB, although producing a similar profile to that obtained after 8 h treatment, evidenced a stronger secretion of TNF-α (2.81±0.45 fold, $P<0.01$) that was maintained at 6 and 7 DIV (2.35±0.37 fold and 2.72±0.39 fold, respectively, $P<0.01$) with a peak levels at day 5 (3.14±0.09 fold, $P<0.01$).

Regarding IL-6, we observed that UCB treatment during 8 h was not able to induce changes in this cytokine secretion when compared with vehicle-treated slices. However, 24 h exposure led to a significant decrease in IL-6 release immediately after UCB treatment and 24 h later (0.32±0.12 fold, $P<0.01$ and 0.38±0.20 fold, $P<0.05$, respectively). In contrast, release of IL-1β and S100B remained unchanged following UCB treatment when compared to vehicle-treated cultures (data not shown).

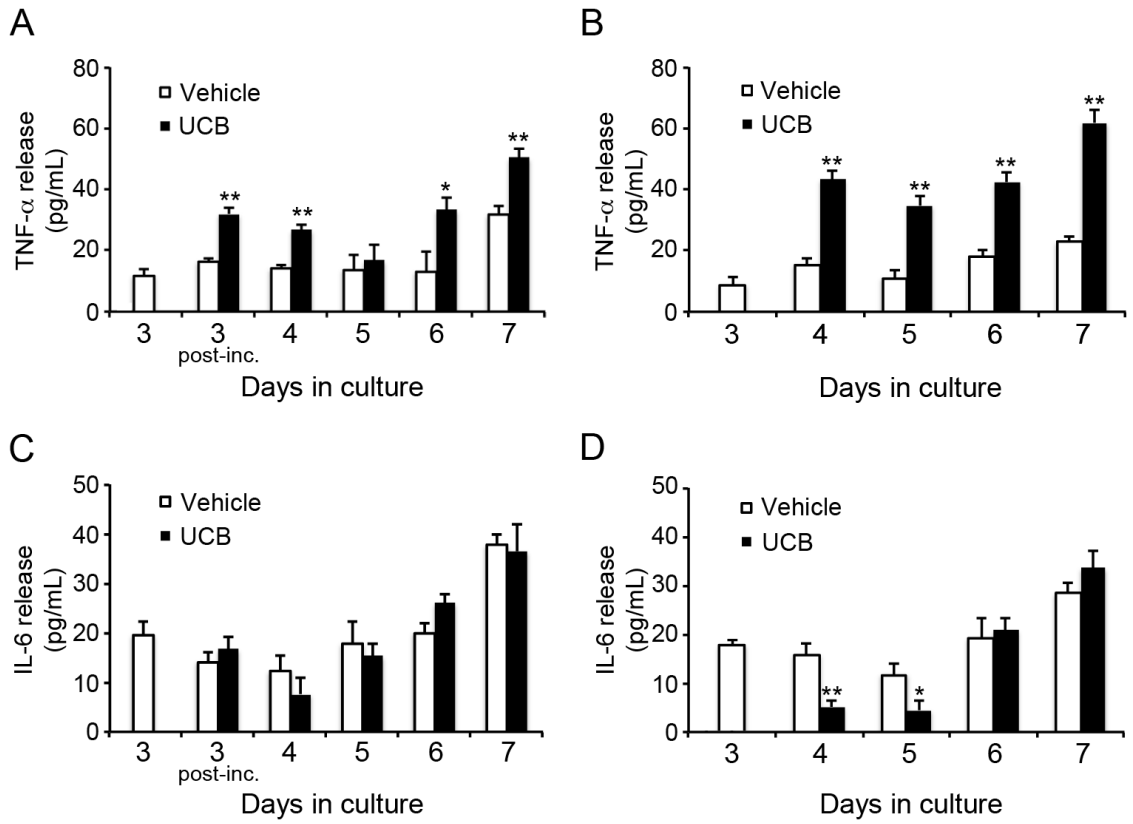


Fig. IV.5 – Unconjugated bilirubin (UCB) leads to an increase in TNF-α secretion and a decrease in that of IL-6. Cerebellar slice cultures were exposed to UCB at 3 day *in vitro* (DIV) for 8 h and 24 h, and maintained in replaced culture medium until 7 DIV. The samples were collected before and after UCB treatment and in the following days, for the determination of TNF-α and IL-6 secretion. Graph bars represent the quantification of TNF-α and IL-6 release after UCB treatment for 8 h (**a**) and (**c**), respectively, as well as at 24 h (**b**) and (**d**), respectively. Results are mean ± SEM from at least five independent experiments performed in duplicate. **P*<0.05 vs. vehicle, ***P*<0.01 vs. vehicle.

3.5. Treatment of cerebellar slice cultures with UCB leads to a fast and transient release of glutamate

Glutamate has been demonstrated to be released from astrocytes, neurons and microglia upon UCB treatment and to be involved in UCB neurotoxicity (Falcão et al. 2006; Fernandes et al. 2004; Gordo et al. 2006; Stevenson et al. 2001). In addition, glutamate is also a known noxious molecule for OL during maturation and myelination (Emery 2010). Hence, we evaluated the accumulation of glutamate in the culture media along slice culture treatment and maintenance, as described before for cytokine determination. UCB-induced secretion of glutamate showed to occur very rapidly and to be highly sensitive to the duration of the exposure (Fig. IV.6). In fact, while the incubation with UCB for 8 h produced a 2.12±0.20 fold (*P*<0.01) immediate increase in the secretion of glutamate into the culture medium, that of 24 h led to a 2.78±0.41 fold enhancement (*P*<0.01). Most striking, glutamate release in more prolonged incubation

with UCB was still noticed in the following 24 h (1.26±0.12 fold, *P*<0.01). Interestingly, it seems that in contrast to the prolonged inflammatory response induced by UCB, glutamate release is a direct effect of UCB only lasting during the time of UCB within the system.

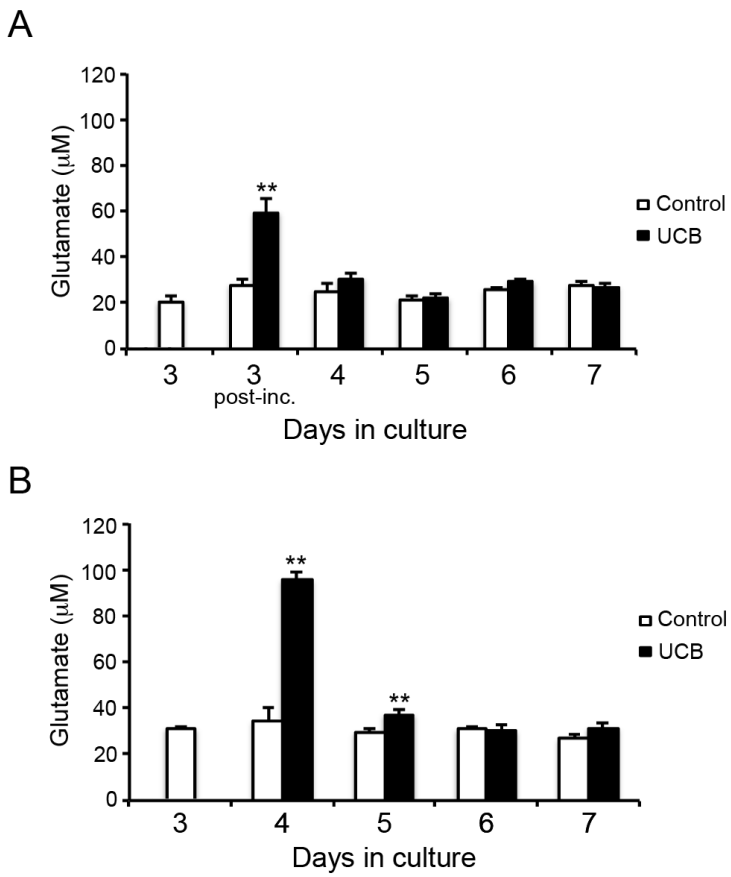


Fig. IV.6 – Unconjugated bilirubin (UCB) induces glutamate release immediately after the treatment period. Cerebellar slice cultures were exposed to UCB at 3 day *in vitro* (DIV) for 8 h and 24 h. After treatment with UCB the slices were maintained until 7 DIV in fresh media. The samples were collected from the culture medium exactly before and after UCB treatment and in the following days for the determination of glutamate release. Graph bars represent the quantification of glutamate release after 8 h (**a**) and 24 h (**b**) of exposure to UCB. Results are mean ± SEM from at least five independent experiments performed in duplicate. ***P*<0.01 vs. vehicle.

4. Discussion

UCB revealed to impair myelination development in our model of moderate to severe neonatal hyperbilirubinemia, by decreasing the number of myelinated fibers and by triggering an elevation of the OL number in an immature stage and thus, unabled to myelinate properly. Myelination occurs in the second week of postnatal development in rodents and in the last trimester of gestation in humans, but is only completed in some brain regions at early adulthood (Rice and Barone 2000). Moreover, myelination is a highly regulated process, closely coordinated between mature OL and neurons, which is essential for normal information processing and learning. Since the disruption of white matter is associated with a wide range of cognitive and psychiatric disorders (Fields 2008), the identification of extrinsic and intrinsic factors that modulate pathophysiological changes by UCB during development is very important to

understand developmental trajectories and provide initial clues for preventive treatment. Dissociated OL cultures and co-cultures with neurons have provided important information on many aspects of these processes. However, these cultures do not allow the maintenance of the tissue cytoarchitecture, as well as the contribution that other glial cells may have on myelination failure. In this context, there is now increasing the interest in the use of organotypic slice cultures for studies on OL biology and myelination due to its retention of multi-cellular interactions and their ease manipulation (Gadea et al. 2009; Yang et al. 2011).

In this study, we started by evaluating if organotypic cerebellar slice cultures treated with UCB evidenced alterations in OL maturation stage and consequent changes in the myelination pattern. In a next step we were interested in evaluate whether those changes, if observed, would be depedent on the duration of UCB treatment. Similarly to what we observed earlier in our *in vitro* models of primary cultures, either that to evaluate OL differentiation or the co-cultures of dorsal root ganglia neurons with OL for myelination assessment (Barateiro et al. 2012b), we found that treatment with UCB increases the number of OPC by inhibiting differentiation, thus causing a decrease in myelination. The effects produced are highly dependent on the time of UCB interaction with the cells, with features that become increasingly noticed as the stimulus lasts.

A recent study using an organotypic slice culture model of chronic white matter injury (Dean et al. 2011) has demonstrated that myelination failure involve the excessive proliferation of OPC and the reduction of differentiated OL. These *in vitro* responses are very similar to those described in neonatal rodents, following hypoxia-ischemia and intermittent hypoxia mimicking apnoea of prematurity, where failure of preoligodendrocytes to maturate and myelination deficits were observed (Cai et al. 2012; Segovia et al. 2008). OL maturation arrest responsibility in myelination deficits is demonstrated by studies in preterm fetal sheep, following hypoxia-ischemia (Riddle et al. 2011), and in preterm human autopsy cases with diffuse white matter injury (Buser et al. 2012). Consequently, the arrest of OL maturation may expand the developmental window of white matter immaturity, which may enhance the risk of brain injuries in neonatal jaundice.

As an individual OL is able to form multiple myelin internodes, myelination is not solely dependent on myelin gene expression. Valuable insights have been produced along the last years by clarifying the transcriptional program necessary for the differentiation of OPC into mature myelinating OL (Emery 2010). In particular, Olig1 and Olig2 have shown to play major roles in defining oligodendroglial lineage, as well as, in the generation and maturation of OL (Ligon et al. 2006a; Ligon et al. 2006b; Zhou

et al. 2000). Olig1 is a transcription factor that regulates myelin gene expression, while Olig2 is implicated in the specification of OPC during development (Copray et al. 2006; Ligon et al. 2004; Lu et al. 2002; Rousseau et al. 2006). In this study, we demonstrated that exposure to UCB determine an increase in Olig2 mRNA expression, but a reduction in that of Olig1. According to our previous results related to OL maturation stage, this parameter also revealed to be influenced by the time of exposure, which correlates with changes in OPC number at both 8 and 24h of treatment when compared with vehicle assay. This hypothesis is in line with studies demonstrating that Olig1 expression peaks during membrane maintenance and is essential for MBP, proteolipid protein (PLP) and myelin associated glycoprotein (MAG) transcription, while that of Olig2 is detected in freshly isolated cells, but is not involved in either outgrowth process or membrane maintenance (Niu et al. 2012; Othman et al. 2011; Xin et al. 2005). Similar findings were observed by our own studies with primary cultures of maturing OL after UCB treatment (Barateiro et al. 2012b) and strong upregulation of Olig2 was also evidenced by Buffo et al. (2005) after acute brain injury and chronic amyloid deposition.

Mounting evidence has shown that astrocytes and microglia besides having several important roles in the developing brain, also impact on CNS damage when brain homeostasis is disrupted (Liu et al. 2011). Therefore, we hypothesized that astrocytes and microglia would contribute to white matter damage upon exposure to UCB and analysed how these cells responded to UCB in cerebellar slice cultures. In this context, we observed an increase in the percentage of area occupied by astrocytes and microglia after UCB treatment when compared with vehicle-treated slices, which indicate an increase in astrogliosis and microgliosis after the insult. Astroglial reactivity was identified in ischemia (Biran et al. 2006), trauma (Rostworowski et al. 1997) and infection (Rousset et al. 2006). Most strikingly, a recent study performed in an organotypic slice culture model of chronic white matter injury has demonstrated a rapid and progressive reactive astrocytosis and microglia accumulation in the white matter of the slice cultures (Dean et al. 2011). Also known is that prenatal ischemia can lead to astrogliosis in several brain regions, such as hippocampus and cortex, in rats with white matter injury (Delcour et al. 2012; Delcour et al. 2011). Additionally, microglial activation was observed during neuroinflammatory events related to the pathogenesis of early periventricular leukomalacia (PVL) (Haynes et al. 2009) and both Iba-1 labelled microglia and astrogliosis were noticed during white matter injury in premature infants (Buser et al. 2012)

In response to injury, both astrocytes and microglia turn into an activated state that entails several features like release of pro-inflammatory cytokines, oxygen radicals

and proteases (Allan and Rothwell 2001; Kim and de Vellis 2005). In line with an increase in astrocytes and microglia cell number, we showed that after UCB treatment there was an accumulation of TNF-α in the supernatant in the course of glia activation. Interestingly, the amount of the cytokine showed to directly increase with the time of incubation indicating the sustained activation of the cells by UCB even when cultured in fresh media in the absence of the pigment. Release of TNF-α was reported to occur in traumatic brain injury (Bermpohl et al. 2007) and demonstrated by us after incubation of pure cultures of microglia and astrocytes with UCB in our model of experimental neonatal jaundice (Fernandes et al. 2004; Gordo et al. 2006). In contrast, UCB produced a fast inhibition of IL-6 secretion that was significant with the 24 h of treatment. This is not without precedent once we have previously demonstrated a similar effect for a short incubation of astrocytes with UCB (Fernandes et al. 2006; Fernandes et al. 2004). Once in microglia, UCB mostly induces IL-1β release (Silva et al. 2010), we may speculate that astrocytes will be the main producers of TNF-α in our cultures of cerebellar slices and that microglia may have adopted a more neuroprotective phenotype, rather than a pro-inflammatory one. Increased production of TNF-α may cause disruption of white matter maturation and injury, as demonstrated by the impairment of myelination in spinal cord-derived culture (Pang et al. 2012) and by hypomyelination in PVL in the presence of high levels of this pro-inflammatory cytokine (Huleihel et al. 2004; Pang et al. 2003). In fact, TNF-α is known to damage OL (Beattie et al. 2010), resulting in deficits in myelin production.

On the other hand, glutamate excitotoxicity has been shown to cause damage to the developing brain in animal models of trauma (Bittigau et al. 1999), as well as in hypoxia/ischemia (Silverstein et al. 1986) and over-activation of non-NMDA glutamate receptors evidenced to trigger OL attenuated lineage progression (Gallo et al. 1996; Yuan et al. 1998). The increased glutamate efflux in our model is in line with previous data indicating that UCB interferes with glutamate uptake in synaptic vesicles (Roseth et al. 1998), astrocytes and neurons (Silva et al. 1999; Silva et al. 2002). Thus, we may hypothesize that alterations produced by UCB treatment in glutamate uptake may have been in the origin of the detrimental effects in OL maturation in our model of moderate hyperbilirubinemia. However, the extracellular increase in glutamate concentration may also be due to an enhanced release, as observed after incubation of astrocytes, neurons and microglia with UCB (Falcão et al. 2006; Fernandes et al. 2004; Gordo et al. 2006). Collectively, we demonstrate that moderate hyperbilirubinemia is able to induce alterations in OL maturation and consequently altered myelination pattern, using an *in vitro* approach that allows the maintenance of nerve and glia cell-cell interactions, as well as of their corresponding milieu. With our results, we can hypothesize that

reduced myelination in jaundiced neonates can derive from OL maturation arrest and impairment of the myelination process, as observed in kernicteric premature infant at autopsy (Brito et al. 2012; Gkoltsiou et al. 2008). In addition, these changes in OL development are probably related with alterations in the transcriptional program of OL, but may also be due to the production of excessive cytokines by other glial cells, as well as to enhanced glutamate release into the extracellular media. Altogether, the *in vitro* model described here emphasizes the concept of a global harmful effect of UCB in CNS resident cells in a three-dimensional scale, but overall that hyperbilirubinemia cause defective OPC differentiation and compromise myelination, features that should be taken in consideration for BIND neuroprotective strategies.

5. References

Allan SM, Rothwell NJ. 2001. Cytokines and acute neurodegeneration. Nature reviews Neuroscience 2(10):734-44.

Barateiro A, Vaz AR, Silva SL, Fernandes A, Brites D. 2012a. ER Stress, Mitochondrial Dysfunction and Calpain/JNK Activation are Involved in Oligodendrocyte Precursor Cell Death by Unconjugated Bilirubin. Neuromolecular medicine, [Epub ahead of print].

Barateiro A, Miron VE, Santos SD, Relvas JB, Fernandes A, ffrench-Constant C, Brites D. 2012b. Arrest of oligodendrocyte differentiation and axonal myelination in an in vitro modelo f hyperbilirubinemia. Submitted.

Beattie MS, Ferguson AR, Bresnahan JC. 2010. AMPA-receptor trafficking and injury-induced cell death. The European journal of neuroscience 32(2):290-7.

Bermpohl D, You Z, Lo EH, Kim HH, Whalen MJ. 2007. TNF alpha and Fas mediate tissue damage and functional outcome after traumatic brain injury in mice. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 27(11):1806-18.

Biran V, Joly LM, Heron A, Vernet A, Vega C, Mariani J, Renolleau S, Charriaut-Marlangue C. 2006. Glial activation in white matter following ischemia in the neonatal P7 rat brain. Experimental neurology 199(1):103-12.

Bittigau P, Sifringer M, Pohl D, Stadthaus D, Ishimaru M, Shimizu H, Ikeda M, Lang D, Speer A, Olney JW and others. 1999. Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. Annals of neurology 45(6):724-35.

Bratlid D. 1990. How bilirubin gets into the brain. Clinics in perinatology 17(2):449-65.

Brites D. 2011. Bilirubin injury to neurons and glial cells: new players, novel targets, and newer insights. Seminars in perinatology 35(3):114-20.

Brites D. 2012. The evolving landscape of neurotoxicity by unconjugated bilirubin: role of glial cells and inflammation. Frontiers in pharmacology 3:88.

Brites D, Brito MA. 2012. Bilirubin toxicity. In: Care of the jaundiced neonate. Stevenson DK, Maisels MJ, Watchko JF. Mc-Graw-Hill Companies, 115-43.

Brito MA, Vaz AR, Silva SL, Falcão AS, Fernandes A, Silva RFM, Brites D. 2010. N-methyl-aspartate receptor and neuronal nitric oxide synthase activation mediate bilirubin-induced neurotoxicity. Molecular medicine 16(9-10):372-80.

Brito MA, Zurolo E, Pereira P, Barroso C, Aronica E, Brites D. 2012. Cerebellar axon/myelin loss, angiogenic sprouting, and neuronal increase of vascular endothelial growth factor in a preterm infant with kernicterus. Journal of child neurology 27(5):615-24.

Buchet D, Baron-Van Evercooren A. 2009. In search of human oligodendroglia for myelin repair. Neuroscience letters 456(3):112-9.

Buffo A, Vosko MR, Erturk D, Hamann GF, Jucker M, Rowitch D, Gotz M. 2005. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. Proc Natl Acad Sci U S A 102(50):18183-8.

Buser JR, Maire J, Riddle A, Gong X, Nguyen T, Nelson K, Luo NL, Ren J, Struve J, Sherman LS and others. 2012. Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. Annals of neurology 71(1):93-109.

Cai J, Tuong CM, Zhang Y, Shields CB, Guo G, Fu H, Gozal D. 2012. Mouse intermittent hypoxia mimicking apnoea of prematurity: effects on myelinogenesis and axonal maturation. The Journal of pathology 226(3):495-508.

Castonguay A, Levesque S, Robitaille R. 2001. Glial cells as active partners in synaptic functions. Progress in brain research 132:227-40.

Chang FY, Lee CC, Huang CC, Hsu KS. 2009. Unconjugated bilirubin exposure impairs hippocampal long-term synaptic plasticity. PloS one 4(6):e5876.

Cohen RS, Wong RJ, Stevenson DK. 2010. Understanding neonatal jaundice: a perspective on causation. Pediatrics and neonatology 51(3):143-8.

Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E. 2006. Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. Stem Cells 24(4):1001-10.

Craig A, Ling Luo N, Beardsley DJ, Wingate-Pearse N, Walker DW, Hohimer AR, Back SA. 2003. Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. Experimental neurology 181(2):231-40.

Dean JM, Riddle A, Maire J, Hansen KD, Preston M, Barnes AP, Sherman LS, Back SA. 2011. An organotypic slice culture model of chronic white matter injury with maturation arrest of oligodendrocyte progenitors. Molecular neurodegeneration 6:46.

Delcour M, Russier M, Amin M, Baud O, Paban V, Barbe MF, Coq JO. 2012. Impact of prenatal ischemia on behavior, cognitive abilities and neuroanatomy in adult rats with white matter damage. Behavioural brain research 232(1):233-44.

Delcour M, Russier M, Xin DL, Massicotte VS, Barbe MF, Coq JO. 2011. Mild musculoskeletal and locomotor alterations in adult rats with white matter injury following prenatal

ischemia. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 29(6):593-607.

Dugas JC, Tai YC, Speed TP, Ngai J, Barres BA. 2006. Functional genomic analysis of oligodendrocyte differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 26(43):10967-83.

Emery B. 2010. Regulation of oligodendrocyte differentiation and myelination. Science 330(6005):779-82.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta neuropathologica 112(1):95-105.

Fernandes A, Falcão AS, Abranches E, Bekman E, Henrique D, Lanier LM, Brites D. 2009. Bilirubin as a determinant for altered neurogenesis, neuritogenesis, and synaptogenesis. Developmental neurobiology 69(9):568-82.

Fernandes A, Falcão AS, Silva RFM, Gordo AC, Gama MJ, Brito MA, Brites D. 2006. Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. Journal of neurochemistry 96(6):1667-79.

Fernandes A, Silva RFM, Falcão AS, Brito MA, Brites D. 2004. Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. Journal of neuroimmunology 153(1-2):64-75.

Fields RD. 2008. White matter in learning, cognition and psychiatric disorders. Trends in neurosciences 31(7):361-70.

Gadea A, Aguirre A, Haydar TF, Gallo V. 2009. Endothelin-1 regulates oligodendrocyte development. The Journal of neuroscience : the official journal of the Society for Neuroscience 29(32):10047-62.

Gallo V, Zhou JM, McBain CJ, Wright P, Knutson PL, Armstrong RC. 1996. Oligodendrocyte progenitor cell proliferation and lineage progression are regulated by glutamate receptor-mediated K+ channel block. The Journal of neuroscience : the official journal of the Society for Neuroscience 16(8):2659-70.

Ghoumari AM, Ibanez C, El-Etr M, Leclerc P, Eychenne B, O'Malley BW, Baulieu EE, Schumacher M. 2003. Progesterone and its metabolites increase myelin basic protein expression in organotypic slice cultures of rat cerebellum. Journal of neurochemistry 86(4):848-59.

Gkoltsiou K, Tzoufi M, Counsell S, Rutherford M, Cowan F. 2008. Serial brain MRI and ultrasound findings: relation to gestational age, bilirubin level, neonatal neurologic status and neurodevelopmental outcome in infants at risk of kernicterus. Early human development 84(12):829-38.

Gordo AC, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Unconjugated bilirubin activates and damages microglia. Journal of neuroscience research 84(1):194-201.

Hanko E, Hansen TW, Almaas R, Paulsen R, Rootwelt T. 2006a. Synergistic protection of a general caspase inhibitor and MK-801 in bilirubin-induced cell death in human NT2-N neurons. Pediatric research 59(1):72-7.

Hanko E, Hansen TW, Almaas R, Rootwelt T. 2006b. Recovery after short-term bilirubin exposure in human NT2-N neurons. Brain research 1103(1):56-64.

Haynes RL, Folkerth RD, Trachtenberg FL, Volpe JJ, Kinney HC. 2009. Nitrosative stress and inducible nitric oxide synthase expression in periventricular leukomalacia. Acta neuropathologica 118(3):391-9.

Huleihel M, Golan H, Hallak M. 2004. Intrauterine infection/inflammation during pregnancy and offspring brain damages: possible mechanisms involved. Reproductive biology and endocrinology : RB&E 2:17.

Kaplan M, Muraca M, Hammerman C, Rubaltelli FF, Vilei MT, Vreman HJ, Stevenson DK. 2002. Imbalance between production and conjugation of bilirubin: a fundamental concept in the mechanism of neonatal jaundice. Pediatrics 110(4):e47.

Kasparov S, Teschemacher AG, Paton JF. 2002. Dynamic confocal imaging in acute brain slices and organotypic slice cultures using a spectral confocal microscope with single photon excitation. Experimental physiology 87(6):715-24.

Kim SU, de Vellis J. 2005. Microglia in health and disease. Journal of neuroscience research 81(3):302-13.

Labombarda F, Gonzalez SL, Lima A, Roig P, Guennoun R, Schumacher M, de Nicola AF. 2009. Effects of progesterone on oligodendrocyte progenitors, oligodendrocyte transcription factors, and myelin proteins following spinal cord injury. Glia 57(8):884-97.

Lai AY, Todd KG. 2008. Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury. Glia 56(3):259-70.

Leite MC, Galland F, Brolese G, Guerra MC, Bortolotto JW, Freitas R, Almeida LM, Gottfried C, Goncalves CA. 2008. A simple, sensitive and widely applicable ELISA for S100B: Methodological features of the measurement of this glial protein. Journal of neuroscience methods 169(1):93-9.

Ligon KL, Alberta JA, Kho AT, Weiss J, Kwaan MR, Nutt CL, Louis DN, Stiles CD, Rowitch DH. 2004. The oligodendroglial lineage marker OLIG2 is universally expressed in diffuse gliomas. Journal of neuropathology and experimental neurology 63(5):499-509.

Ligon KL, Fancy SP, Franklin RJ, Rowitch DH. 2006a. Olig gene function in CNS development and disease. Glia 54(1):1-10.

Ligon KL, Kesari S, Kitada M, Sun T, Arnett HA, Alberta JA, Anderson DJ, Stiles CD, Rowitch DH. 2006b. Development of NG2 neural progenitor cells requires Olig gene function. Proceedings of the National Academy of Sciences of the United States of America 103(20):7853-8.

Liu W, Tang Y, Feng J. 2011. Cross talk between activation of microglia and astrocytes in pathological conditions in the central nervous system. Life sciences 89(5-6):141-6.

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 109(1):75-86.

Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH. 2000. Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron 25(2):317-29.

McDonagh AF, Assisi F. 1972. The ready isomerization of bilirubin IX- in aqueous solution. The Biochemical journal 129(3):797-800.

Nave KA. 2010. Myelination and the trophic support of long axons. Nature reviews Neuroscience 11(4):275-83.

Nicolay DJ, Doucette JR, Nazarali AJ. 2007. Transcriptional control of oligodendrogenesis. Glia 55(13):1287-99.

Niu J, Mei F, Wang L, Liu S, Tian Y, Mo W, Li H, Lu QR, Xiao L. 2012. Phosphorylated olig1 localizes to the cytosol of oligodendrocytes and promotes membrane expansion and maturation. Glia.

Othman A, Frim DM, Polak P, Vujicic S, Arnason BG, Boullerne AI. 2011. Olig1 is expressed in human oligodendrocytes during maturation and regeneration. Glia 59(6):914-26.

Pang Y, Cai Z, Rhodes PG. 2003. Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. Brain research Developmental brain research 140(2):205-14.

Pang Y, Zheng B, Kimberly SL, Cai Z, Rhodes PG, Lin RC. 2012. Neuron-oligodendrocyte myelination co-culture derived from embryonic rat spinal cord and cerebral cortex. Brain and behavior 2(1):53-67.

Rice D, Barone S, Jr. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environmental health perspectives 108 Suppl 3:511-33.

Riddle A, Dean J, Buser JR, Gong X, Maire J, Chen K, Ahmad T, Cai V, Nguyen T, Kroenke CD and others. 2011. Histopathological correlates of magnetic resonance imaging-defined chronic perinatal white matter injury. Annals of neurology 70(3):493-507.

Roseth S, Hansen TW, Fonnum F, Walaas SI. 1998. Bilirubin inhibits transport of neurotransmitters in synaptic vesicles. Pediatric research 44(3):312-6.

Rostworowski M, Balasingam V, Chabot S, Owens T, Yong VW. 1997. Astrogliosis in the neonatal and adult murine brain post-trauma: elevation of inflammatory cytokines and the lack of requirement for endogenous interferon-gamma. The Journal of neuroscience : the official journal of the Society for Neuroscience 17(10):3664-74.

Rousseau A, Nutt CL, Betensky RA, Iafrate AJ, Han M, Ligon KL, Rowitch DH, Louis DN. 2006. Expression of oligodendroglial and astrocytic lineage markers in diffuse gliomas: use of YKL-40, ApoE, ASCL1, and NKX2-2. Journal of neuropathology and experimental neurology 65(12):1149-56.

Rousset CI, Chalon S, Cantagrel S, Bodard S, Andres C, Gressens P, Saliba E. 2006. Maternal exposure to LPS induces hypomyelination in the internal capsule and programmed cell death in the deep gray matter in newborn rats. Pediatric research 59(3):428-33.

Schnadelbach O, Ozen I, Blaschuk OW, Meyer RL, Fawcett JW. 2001. N-cadherin is involved in axon-oligodendrocyte contact and myelination. Molecular and cellular neurosciences 17(6):1084-93.

Segovia KN, McClure M, Moravec M, Luo NL, Wan Y, Gong X, Riddle A, Craig A, Struve J, Sherman LS and others. 2008. Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. Annals of neurology 63(4):520-30.

Shapiro SM. 2005. Definition of the clinical spectrum of kernicterus and bilirubin-induced neurologic dysfunction (BIND). Journal of perinatology : official journal of the California Perinatal Association 25(1):54-9.

Silva RFM, Mata LR, Gulbenkian S, Brito MA, Tiribelli C, Brites D. 1999. Inhibition of glutamate uptake by unconjugated bilirubin in cultured cortical rat astrocytes: role of concentration and pH. Biochemical and biophysical research communications 265(1):67-72.

Silva RFM, Rodrigues CM, Brites D. 2002. Rat cultured neuronal and glial cells respond differently to toxicity of unconjugated bilirubin. Pediatric research 51(4):535-41.

Silva SL, Osorio C, Vaz AR, Barateiro A, Falcão AS, Silva RFM, Brites D. 2011. Dynamics of neuron-glia interplay upon exposure to unconjugated bilirubin. Journal of neurochemistry 117(3):412-24.

Silva SL, Vaz AR, Barateiro A, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2010. Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. Neurobiology of disease 40(3):663-75.

Silverstein FS, Buchanan K, Johnston MV. 1986. Perinatal hypoxia-ischemia disrupts striatal high-affinity [3H]glutamate uptake into synaptosomes. Journal of neurochemistry 47(5):1614-9.

Stevenson DK, Dennery PA, Hintz SR. 2001. Understanding newborn jaundice. Journal of perinatology : official journal of the California Perinatal Association 21 Suppl 1:S21-4; discussion S35-9.

Volterra A, Meldolesi J. 2005. Astrocytes, from brain glue to communication elements: the revolution continues. Nature reviews Neuroscience 6(8):626-40.

Xin M, Yue T, Ma Z, Wu FF, Gow A, Lu QR. 2005. Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. J Neurosci 25(6):1354-65.

Yang Y, Lewis R, Miller RH. 2011. Interactions between oligodendrocyte precursors control the onset of CNS myelination. Developmental biology 350(1):127-38.

Yuan X, Eisen AM, McBain CJ, Gallo V. 1998. A role for glutamate and its receptors in the regulation of oligodendrocyte development in cerebellar tissue slices. Development 125(15):2901-14.

Zhang H, Jarjour AA, Boyd A, Williams A. 2011. Central nervous system remyelination in culture--a tool for multiple sclerosis research. Experimental neurology 230(1):138-48.

Zhou Q, Anderson DJ. 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109(1):61-73.

Zhou Q, Wang S, Anderson DJ. 2000. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron 25(2):331-43.

Chapter V

REDUCED MYELINATION AND INCREASED GLIA REACTIVITY IN A HYPERBILIRUBINEMIC MOUSE MODEL

Andreia Barateiro[1], Adelaide Fernandes[1,2], Helena S. Domingues[3], João B. Relvas[3,4], Shujuan Chen[5], Robert Tukey[5], Dora Brites[1,2]

[1]Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL),
Phaculty of Pharmacy, University of Lisbon,
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

[2]Department of Biochemistry and Human Biology,
Faculty of Pharmacy, University of Lisbon
Av. Professor Gama Pinto, 1649-003 Lisbon, Portugal

[3]Instituto de Biologia Molecular e Celular, University of Porto
Rua do Campo Alegre 823, 4150-180 Porto, Portugal

[4]Departamento de Biologia Experimental,
Faculty of Medicine, University of Porto
Av. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

[5]Department of Pharmacology, and Chemistry & Biochemistry
University of California, San Diego
9500 Gilman Drive MC 0722, La Jolla, CA 92093-0722, USA

(in preparation)

Acknowledgments

This work was supported by the strategic project PEst-OE/SAU/UI4013/2011 and PTDC/SAU-NEU/64385/2006 grants from Fundação para a Ciência e a Tecnologia (FCT), Lisbon, Portugal (to D. B.). A. B. was recipient of a PhD fellowship (SFRH/BD/43885/2008) from FCT. H.S.D. is supported by a fellowship from European Commission (Marie Curie IEF 2010, #276322). Work in the lab of JBR was supported by a PTDC/BIA-BCM/112730/2009 from FCT. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Bilirubin-induced neurologic dysfunction (BIND) or *kernicterus* have been used to describe moderate to severe neurological dysfunction observed in children exposed to excessive levels of unconjugated bilirubin (UCB) during the neonatal period. To date the most used animal model to study this pathophysiological condition was the Gunn rat model, which upon sulphadimethoxine treatment, to acutely displace UCB from albumin, mimic the effects of acute hyperbilirubinemia in jaundiced newborns. Here we use a new mice model of *kernicterus* to assay central nervous system alterations concerning myelination and glia reactivity. *Kernicterus* mice brains displayed a diffuse yellow coloration and a marked cerebellum hypoplasia. Histologically, hyperbilirubinemic mice showed axonopathy with myelination deficits at different brain regions including pons, medulla oblongata and cerebellum. These changes were accompanied with increased astroglial and microglial reactivity, possibly as a response to myelination injury. Interestingly, cerebellum was the most affected area with greater myelination impairment and glia burden, showing also a marked loss of Purkinje cells and a reduced arborization of the remaining ones. Collectively, we present a new animal model of *kernicterus* that display not only axonal damage but also myelination deficits and gliosis in different brain regions that are usually related to the neurological *sequelae* observed in *kernicterus* cases.

Keywords: Hyperbilirubinemia mouse model, myelination, microglia and astrocytes reactivity, cerebellum

1. Introduction

Hyperbilirubinemia is a highly common clinical phenomenon occurring in the neonatal period. Indeed, over 60% of term and virtually all premature infants experience temporary, mild to moderate “physiological” jaundice, due to excessive production of unconjugated bilirubin (UCB) and immaturity of hepatic conjugation with consequent impaired UCB clearance (Stevenson et al. 2001; Cohen et al. 2010). Although in the vast majority of cases neonatal jaundice represents a benign condition, in some newborns the concentration of UCB may rise to higher levels causing the syndrome of bilirubin-induced neurologic dysfunction (BIND) or may progress to *kernicterus* resulting in severe neurological dysfunctions (Johnson and Bhutani 2011). Hence, we and others have been focused on understanding the molecular mechanisms by which UCB exerts such neurodevelopmental abnormalities.

Several *in vitro* models have shown that UCB is neurotoxic, promoting nerve cell demise in a concentration- and time-dependent manner (Hanko et al. 2005; Fernandes et al. 2004; Silva et al. 2010; Gordo et al. 2006; Barateiro et al. 2012a; Falcão et al. 2006). Mechanistically, moderate levels of UCB promote accumulation of extracellular glutamate (Fernandes et al. 2004; Falcão et al. 2006; Gordo et al. 2006; Falcão et al. 2005) and excitotoxicity (Grojean et al. 2001; McDonald et al. 1998; Brito et al. 2010), as well as oxidative stress (Barateiro et al. 2012a; Vaz et al. 2010), inducing intracellular mitochondria dysfunction (Barateiro et al. 2012a; Vaz et al. 2010), endoplasmic reticulum stress (Barateiro et al. 2012a) and caspase activation (Barateiro et al. 2012a; Vaz et al. 2010; Silva et al. 2010) culminating in cell death. Interestingly, we also showed that UCB is able to stimulate astrocytes and microglia into an inflammatory response with cytokine release (Gordo et al. 2006; Fernandes et al. 2006; Fernandes et al. 2004; Silva et al. 2011) through activation of mitogen-protein kinases and nuclear factor kB signalling cascades (Silva et al. 2010; Fernandes et al. 2006). Curiously, by depleting microglia with clodronate liposomes in an organotypic hippocampal slice system, our group have also demonstrated that microglia is responsible, at least in part, for glutamate and nitric oxide release after UCB exposure, as well as for consequent neuritic impairment and cell death (Silva et al. 2012). More recently, we demonstrated that also oligodendrocyte maturation and myelination is impaired by UCB either in *in vitro* dorsal-root ganglia neuron-oligodendrocyte co-cultures (Barateiro et al. 2012b) and organotypic cerebellar slice cultures (Barateiro et al. 2012c) or in a *kernicterus* case of a premature infant (Brito et al. 2012).

In situations of increased concentrations of UCB in the brain, specific regions of the central nervous system (CNS) are particularly susceptible to UCB toxicity, such as

the basal ganglia, central and peripheral auditory pathways, hippocampus, diencephalon, subthalamic nuclei, midbrain, pontine and brain stem nuclei for oculomotor function, respiratory, neurohumoral and electrolyte control, and the cerebellum (Bhutani and Stevenson 2011). Consequently the clinical features of UCB encephalopathy may range from subtle multisensory and learning/cognitive impairments during infancy to deafness and severe cerebral palsy, seizure or death in *kernicterus* (Johnson and Bhutani 2011; Shapiro 2010). Hence, considerable interest is focused on understanding how UCB exerts such neurodevelopmental abnormalities in a more precise experimental model, such as an animal model.

One of the most used animal model for UCB encephalopathy is the Gunn rat that is a mutant of the Wistar strain (Gunn 1944). The Gunn rat has a genetic deficiency in glucoronyl transferase that is transmitted as an autosomal recessive trait. The normal homozygote animal presents the enzyme with complete activity, the heterozygote (Jj) has approximately 50% enzyme activity and the homozygous recessive animal (jj) lacks the enzyme. Moreover, some homozygous recessive rats present same neurological symptoms and histopathological lesions that are exhibited by hyperbilirubinemic human newborns (Schutta and Johnson 1969). In addition, it has been used the treatment of jj Gunn rats with sulphadimethoxine in order to acutely displace UCB from albumin and mimic the effects of acute increases in UCB that can occur in jaundiced newborns (Gazzin et al. 2008). Histologically, the most marked effect presented by jj Gunn rats was definitely a marked cerebellar hypoplasia (Conlee and Shapiro 1997). At cellular levels it was reported an increase in the number of dense bodies in astrocytes and oligodendrocytes with no myelination deficits (Sturrock and Jew 1978), and hypertrophy of the fibrous astrocytes without glial hyperplasia (Mikoshiba et al. 1980). Others indicate increased number of microglia-like cells in the severe affected cerebellar lobules (Keino et al. 1986), being recently also detected increased microglia activation in Gunn rat hippocampus without hyperplasia (Liaury et al. 2012). On the other hand, others also showed altered neurotypic and gliotypic proteins, namely a decrease in myelin basic protein (MBP) (O'Callaghan and Miller 1985), with changes in myelin figures observed by electron microscopy (Jew et al. 1977). In parallel it was also reported a decrease of Purkinje cell density in jj Gunn rats (O'Callaghan and Miller 1985), showing increased cytoplasmic vacuolation followed by necrotic degeneration (Conlee and Shapiro 1997).

Here, we used a new model of severe hyperbilirubinemia, created by Robert Tukey and co-workers, to examine resulting CNS changes concerning myelination and glial reactivity from UCB-induced toxicity. These mice mimic the effects of acute and severe increase in UCB that can occur in jaundiced newborns, which may result in

irreversible *kernicterus*. Hyperbilirubinemic mice develop, without any further treatment, increased bilirubin levels during the first week of life and present seizures leading, later on, to 90% of animal death. This condition results in a diffuse yellow coloration of mice brains with a reduced cerebellum volume. Microscopically hyperbilirubinemic mice present reduced number of axons in parallel with reduced myelination at different brain regions including pons, medulla oblongata and cerebellum and no change at corpus callosum. In parallel, myelination impairment was associated with increased astroglial and microglial reactivity in the same brain regions. Interestingly, cerebellum was the most affected area with greater myelination impairment at the white matter terminals but an increased glial reactivity at white matter ramifications, possibly indicating that glia may be activated to rescue myelination impairment. To note also Purkinje cells were highly affected in hyperbilirubinemic mice, presenting a reduced number and a less ramified morphology. Overall the data presented here show that severe hyperbilirubinemia markedly affect not only axonal formation but also myelination in different brain regions, with a more pronounced effect at the cerebellum which may account for the *sequelae* observed in *kernicterus* cases.

2. Material and Methods

2.1. Animals

The present model was used in collaboration with Robert Tukey under a confidentiality agreement and will be further disclosed upon animal characterization. To the present it is known that these pups present marked increased levels of UCB without any further treatment and develop seizures culminating in animal death during the first post-natal week. All procedures were performed with the approval of the University of California, San Diego, Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of USA.

2.2. Immunohistochemistry

Mice were anesthetized with pentobarbital (40 mg/Kg, i.p.) and perfused with 0.1 M phosphate buffer saline (PBS) (pH 7.4) followed by the same buffer containing 4% paraformaldehyde (PFA). Fixed tissues were post-fixed in 4% PFA in PBS for 72 h at room temperature (RT), dehydrated through a graded ethanol series and embedded in paraffin.

For immunostaining, 3 μm sections were submitted to antigen retrieval in 20 mM citrate buffer with 1.5% H_2O_2 for 15 min at RT in the dark, incubated for 10 min in Tris/EDTA buffer at 84°C and blocked for 1 h at RT in 1% bovine serum albumin (BSA) in PBS. Primary antibodies, mouse anti-neurofilaments-medium (AbCam #ab7794, 1:50), rat anti-myelin basic protein (MBP) (AbDSerotec #MCA4095, 1:50) for oligodendrocyte and myelination detection, rabbit anti-glial fibrillary acidic protein (GFAP) (Sigma #G9269, 1:250) for astrocyte detection and rabbit anti-ionized calcium binding adaptor molecule (Iba)-1 (Wako #019-19741, 1:250) for microglial staining, were used in 0.5% BSA in PBS overnight at 4 °C. For Purkinje cells staining, sections were blocked for 2 h at RT in 10% normal goat serum and 0.5% Triton X-100 in PBS and incubated overnight with mouse anti Adenomatus Polyposis Coli (CC-1) (Merck, #OP80, 1:100) in 2% goat serum and 0.3% Triton X-100 in PBS. After washing in PBS, sections were incubated for 1 h at RT with antibodies anti-mouse coupled to AlexaFluor 488 (Invitrogen #A11001, 1:1,000) or anti-goat IgG (H+L) Cy3-conjugated (Jackson ImmunoResearch, #115-166-003, 1:3,000), anti-rat coupled to AlexaFluor 568 (Invitrogen #A11077, 1:1,000) or to AlexaFluor 488 (Invitrogen #A11006, 1:1,000), and anti-rabbit coupled to Alexafluor 568 (Invitrogen #A11036, 1:1,000) in 0.5% BSA in PBS, incubated for 20 min in DAPI and mounted with Shandon Immu-Mount™ Aqueous Non-fluorescing Mounting Medium (Thermo Scientific).

Tissue sections were visualized in an AxioImager Z1 fluorescence microscope equipped with 10x/0.30 Ph1, 20x/0.50 Ph2 and 40x/1.30 Oil Ph3 EC-Plan-Neofluar objectives (Carl Zeiss, Germany). Images were acquired with MRm Axiocam ver 3.0 camera connected to a PC running the Axiovision 4 acquisition software (Carl Zeiss, Germany).

2.3. Measuring myelination and counting Iba-1 and GFAP-labelled cells

In order to evaluate if *kernicterus* mice present alterations in brain morphology, axonal viability and myelination along several brain regions, sections were stained with neurofilament and MBP. To evaluate overall neuronal and myelination alterations, 10x magnification images were acquired and stitched using Microsoft Image Composite Editor. Then binary masks were specified in ImageJ software using the same cut-off intensity threshold value for each region of interest, defined as the minimum intensity due to specific staining above background values. Finally, the percentage of area immunoreactive for neurofilament and MBP was measured. In addition, it was also calculated the percentage of myelinated fibers obtained by the ratio of MBP to neurofilaments staining. Moreover, changes in brain morphology were identified

measuring the percentage of brain area occupied by cerebellum using the same software.

In order to evaluate more closely changes in myelination in the different brain areas, the percentage of area immunoreactive for neurofilament and MBP was measured in 40x magnification images, using binary masks as described before, in different brain regions (medulla oblongata, pons, cerebellum and corpus callosum) and in specific regions of cerebellum (white matter nodes, middle of white matter tracts and white matter terminals).

Regarding to the evaluation of microglia and astrocytes, we determined the number of cells with positive staining for Iba-1 and GFAP, respectively, in the same regions that we evaluated myelination alterations. Moreover, we have also determined the number of Purkinje cells per cerebellum, identified by CC1+ staining.

All results were given by averaging values determined in at least seven separate microscopic fields from three different sections from two control animals and three *kernicterus* animals. Values are expressed as mean ± SEM.

2.4. Statistical analysis

All results are presented as mean ± SEM. Significant differences between two groups were determined by the two-tailed *t*-test performed on the basis of equal and unequal variance as appropriate and the P-values of $*P<0.05$ and $**P<0.01$ were considered as statistically significant.

3. Results

3.1. Alterations in brain morphology of *kernicterus* mice

Upon brain isolation from the animals it could be easily observed a diffuse yellow staining throughout the brain of the *kernicterus* mice and a reduced cerebellum volume, as previously indicated in the Gunn rat model (Conlee and Shapiro 1997). Then when sagittal brain sections were immunostained, as demonstrated in Fig. V.1, it become clear the marked reduction of the cerebellum when compared with the control mice. Interestingly, when we take a closer look it seems that the global brain area is not affected, possibly due to an increased area of the cortex and midbrain that in the *kernicterus* case appears on top of the cerebellum.

3.2. *Kernicterus* mice present axonal loss and decreased myelination

Given our recent data on the effect of UCB on myelination (Barateiro et al. 2012a,b), we first decided to evaluate if the *kernicterus* mice would present neuronal loss and changes in myelination. So, brain sections were immunostained for neurofilaments and MBP to calculate the percentage of brain area occupied by these markers. As depicted in Fig. V.1, we observed a decrease in brain area occupied by neurofilaments staining (0.80±0.08 fold, $P<0.05$) in the *kernicterus* mice when compared with control mice. Curiously, when we look at changes related with myelination we observed a more pronounced decrease in MBP+ staining along all brain regions (0.62±0.04 fold, $P<0.01$) and in the percentage of myelinated fibers (0.74±0.04 fold, $P<0.05$) in the *kernicterus* animals. So, these results indicate that besides *kernicterus* mice present a reduced number of viable axons, myelination impairment also occurs probably due to a decreased ability of OL to myelinate.

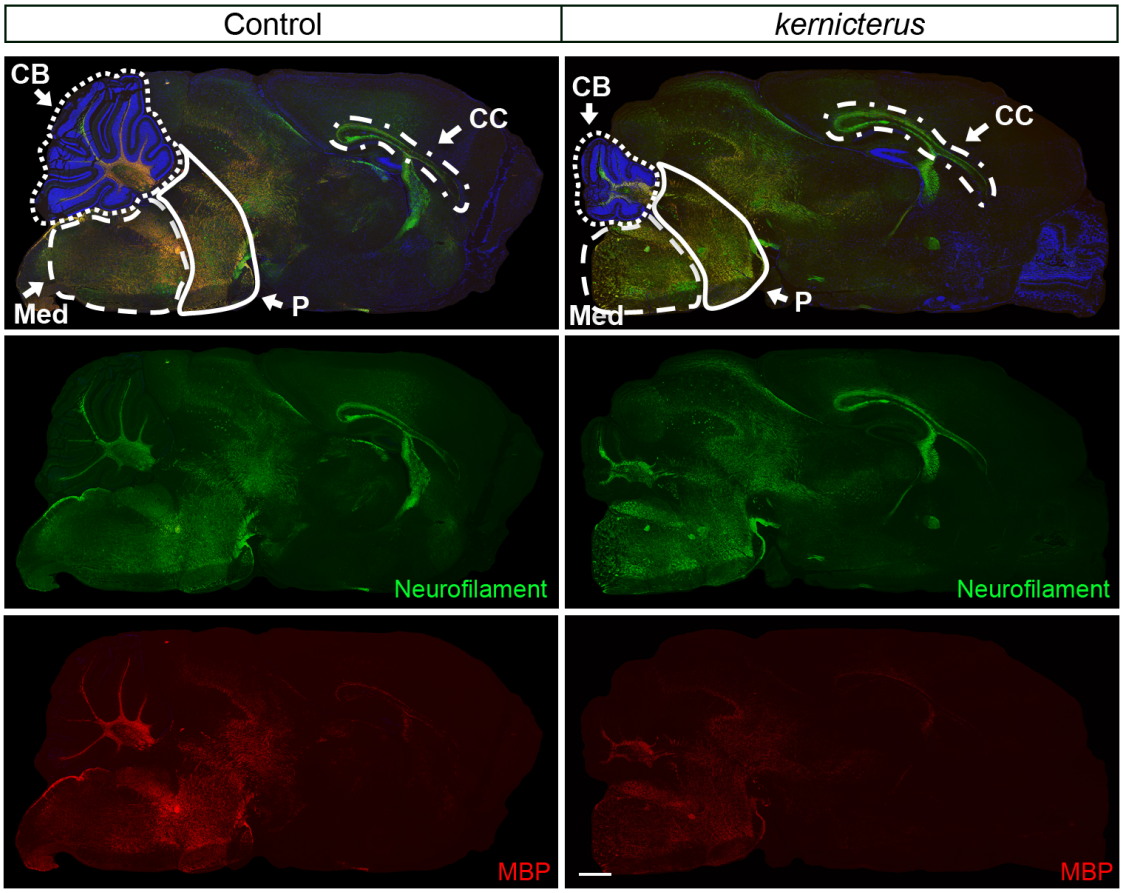


Fig. V.1 – *Kernicterus* mice present axonal loss and decreased myelination. Serial sagittal images of control and *kernicterus* mice. Each image represents a montage of 100-150 images at 10x magnification. 3 μm brain sections of each animal were immunolabelled to identify neuronal axons (neurofilament, green, middle panel) and myelin basic protein (MBP, red, bottom panel). Top panel represents the superposition of neurofilaments, MBP and DAPI staining, the last used for nuclei counterstain (blue). Scale bar represents 700 μm.

3.3. *Kernicterus* mice present reduced myelination and increased glial reactivity in cerebellum, medulla oblongata and pons

As we perceived changes in myelination along different brain regions we decided to observe more closely some brain regions that present myelination in mice at postnatal day 7 and are usually described as affected areas during severe hyperbilirubinemia. So, we evaluated changes in myelination in medulla oblongata, pons, cerebellum and corpus callosum, the last as a positive control of myelination since it is one of the first myelinated areas of the brain (Sturrock 1980). Regarding myelination, as depicted in Figs. V.2a and V.3a, we observed a decrease in the percentage of myelinated fibers in medulla oblongata (0.81±0.03 fold, $P<0.01$), and a more marked effect in pons (0.58±0.03 fold, $P<0.01$) and cerebellum (0.61±0.04 fold, $P<0.01$), with no effect on corpus callosum. Taking a closer look at the images we can also see that while in the control animal the myelinated fibers appear as long and thin strait lines, in the *kernicterus* mice the MBP staining appears as fragmented fibers and myelin agglomerates, suggesting a destruction of the myelin sheath surrounding the axons. Interestingly, the corpus callosum maintains the long myelinated fibers even in the *kernicterus* mice, corroborating no myelin changes.

In our previous study using organotypic cerebellar slice cultures, we have demonstrated that UCB treatment is able to promote astrogliosis and microgliosis (Barateiro et al. 2012c). In other hand, it is known that astrocytes (Zhang et al. 2006) and microglia (Olah et al. 2012) cooperate to create a favourable environment for myelination, while upon myelination injury they are rapidly activated (Petzold et al. 2002). So we next evaluated changes in microglia and astrocyte reactivity in the *kernicterus* cases, identified by Iba-1 and GFAP staining, respectively. As shown in Fig. V.2, the *kernicterus* mice present a marked increase in glia number, both microglia and astrocytes in cerebellum, medulla oblongata and pons with no effect in the corpus callosum. Regarding microglia (Figs. V.2b and V.3b), the major increase was observed in cerebellum (3.75±0.04 fold, $P<0.01$), followed by pons (2.22±0.07 fold, $P<0.01$) and medulla oblongata (1.68±0.21 fold, $P<0.05$). Concerning astrocytes, very similar increases were observed in medulla oblongata and cerebellum (3.73±0.17 fold, $P<0.05$ and 3.95±0.08 fold, $P<0.01$, respectively), but the most pronounced effects were noticed in pons (29.08±3.93 fold, $P<0.01$). Regarding the corpus callosum, we can see that this region has already a considerable number of glial cells also in the control animals presenting no noticeable changes in the *kernicterus* mice.

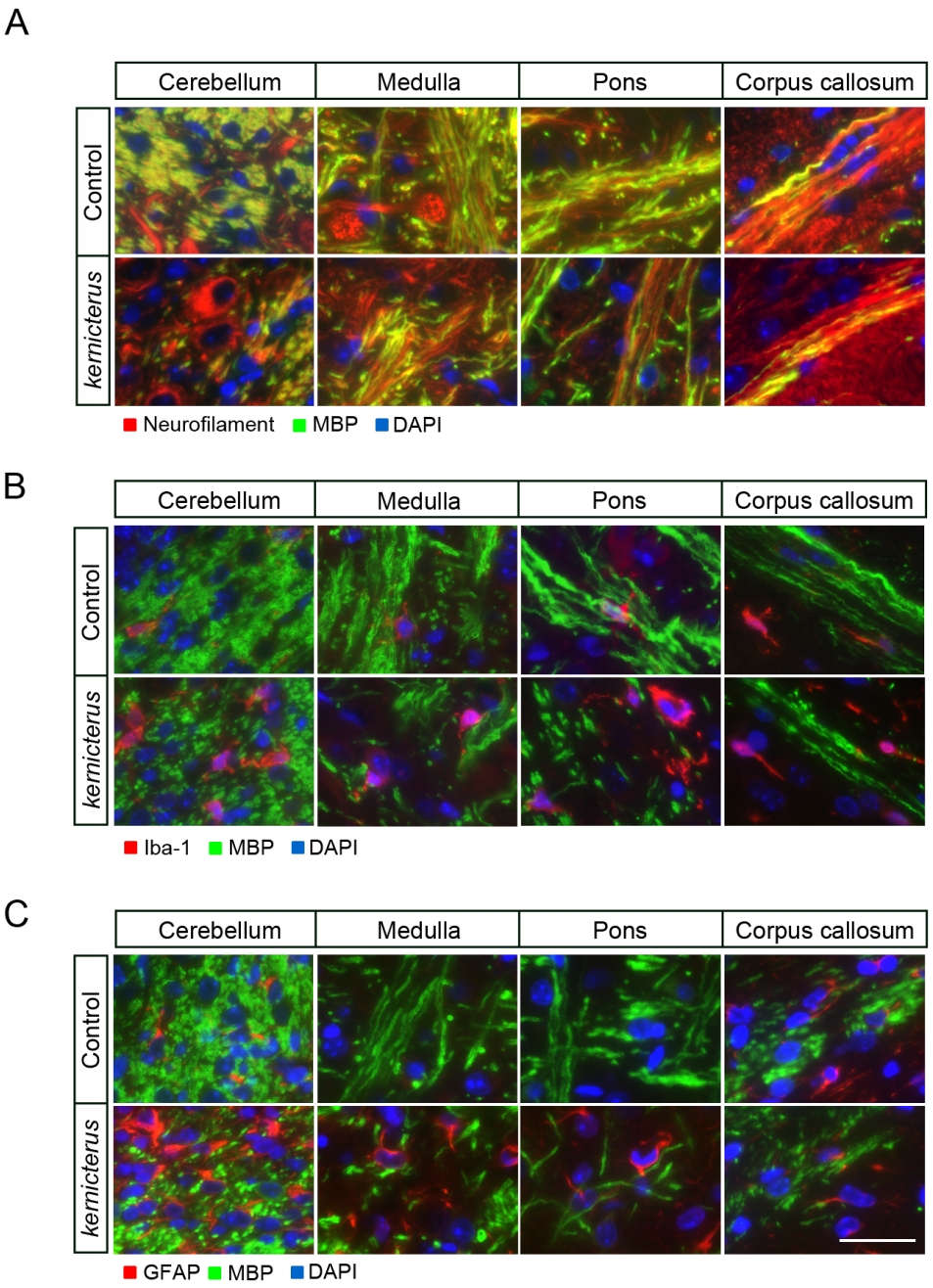


Fig. V.2 – Reduced myelination and increased glial reactivity were observed in *kernicterus* mice, in specific regions, such as cerebellum, medulla oblongata and pons. Representative images from cerebellum, medulla oblongata and corpus callosum from control and *kernicterus* mice immunolabelled to identify **a** neurons (neurofilament, red) and myelin basic protein (MBP, green), **b** microglia (ionized calcium binding adaptor molecule, Iba-1+ staining, red) and MBP (green) and **c** astrocytes (glial fibrillary acidic protein, GFAP+ staining, red) and MBP (green). Nuclei were counterstained with DAPI dye (blue). Scale bar represents 25 μm.

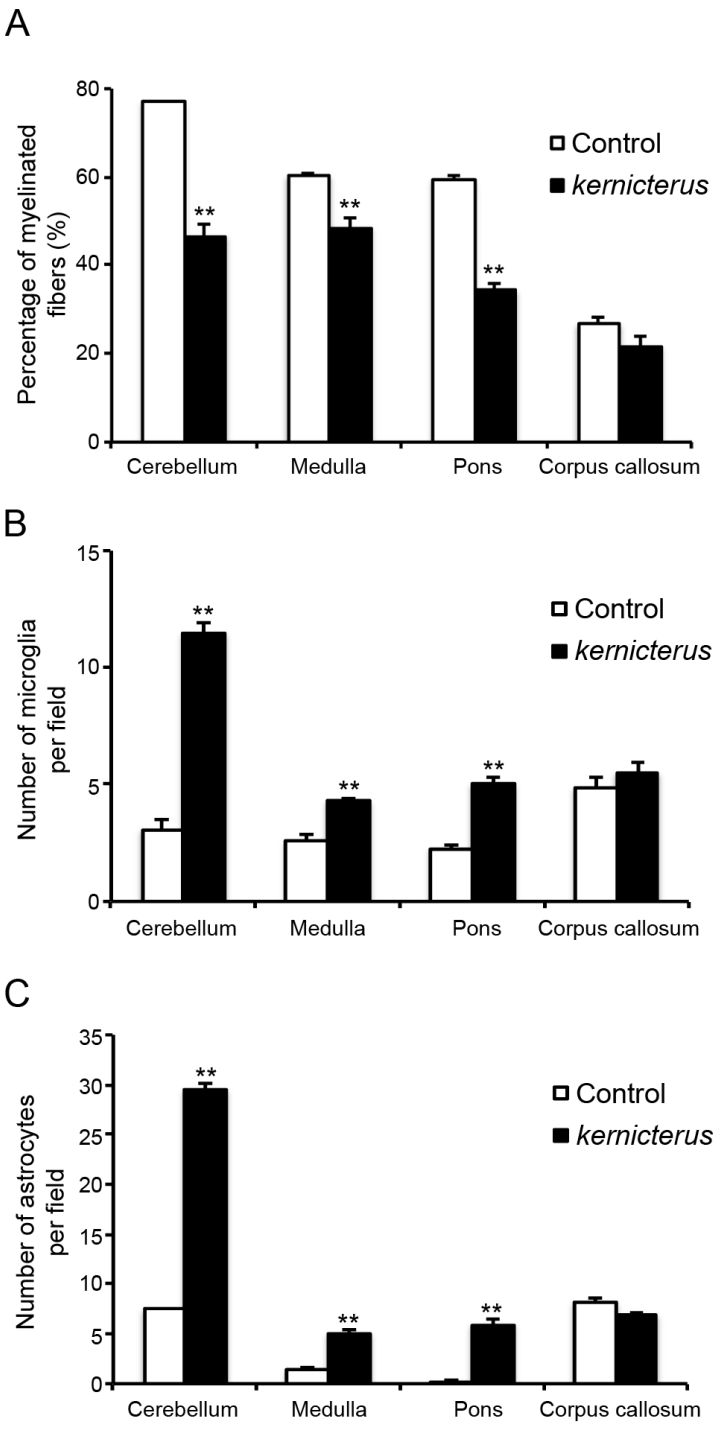


Fig. V.3 – Reduced myelinated fibers and increased microglia and astroglia burden in the *kernicterus* mice, in specific regions, such as cerebellum, medulla oblongata and pons. Graph bars represents the quantification of percentage of myelinated fibers (**a**) and the number of microglia (**b**) and astrocyte (**c**) per field in cerebellum, medulla oblongata, pons and corpus callosum from control and *kernicterus* mice. Results are mean ± SEM from two control mice and three *kernicterus* mice performed in triplicate. ***P*<0.01 vs. respective control.

3.4. Distinct white matter regions from cerebellum present different alterations in myelination and glial reactivity

Since cerebellum is one of the most affected areas and it seems to present regional changes in myelination and glial reactivity, we decided to analyse different areas within the white matter tracts. So, we divided the cerebellum in three distinct zones as depicted in Fig. V.4a, the white matter nodes (region 1), the middle of the white matter tracts (region 2) and white matter terminals (region 3). When myelination was evaluated (Figs. V.4b and V.5a) we observed a marked decrease in the percentage of myelinated fibers in the *kernicterus* mice. The major effects were observed at white matter terminals (0.45±0.07 fold, $P<0.01$), followed by central white matter tracts (0.58±0.04 fold, $P<0.01$) and finally, a less pronounced but significant alteration was observed in white matter nodes (0.72±0.05 fold, $P<0.05$).

Next we also evaluated the number of glia in these regions and observed a marked increase in both astrocyte and microglia number in cerebellum white matter of *kernicterus* mice (Figs. V.4c, d and V.5b, c). In this context, it was observed increased numbers of microglia in all three regions with a similar pattern to what was observed for myelination. Indeed, the most affected area was the white matter terminals (5.37±0.18 fold, $P<0.01$), followed by central white matter tracts (5.05±0.27 fold, $P<0.01$) and white matter nodes (3.11±0.17 fold, $P<0.01$). On the other hand, when astrocyte number was evaluated, *kernicterus* mice presented a very similar increase in both white matter nodes and terminals (3.36±0.18 fold and 3.31±0.25 fold, respectively, $P<0.01$), being the most affected area the central white matter (4.28±0.15 fold, $P<0.01$).

It deserves to be noted that while white matter terminals was the most affected area regarding to myelination degree and microgliosis, astrogliosis was mainly observed in the middle of white matter tracts, possibly suggesting a different role for microglia and astrocytes in response to UCB injury.

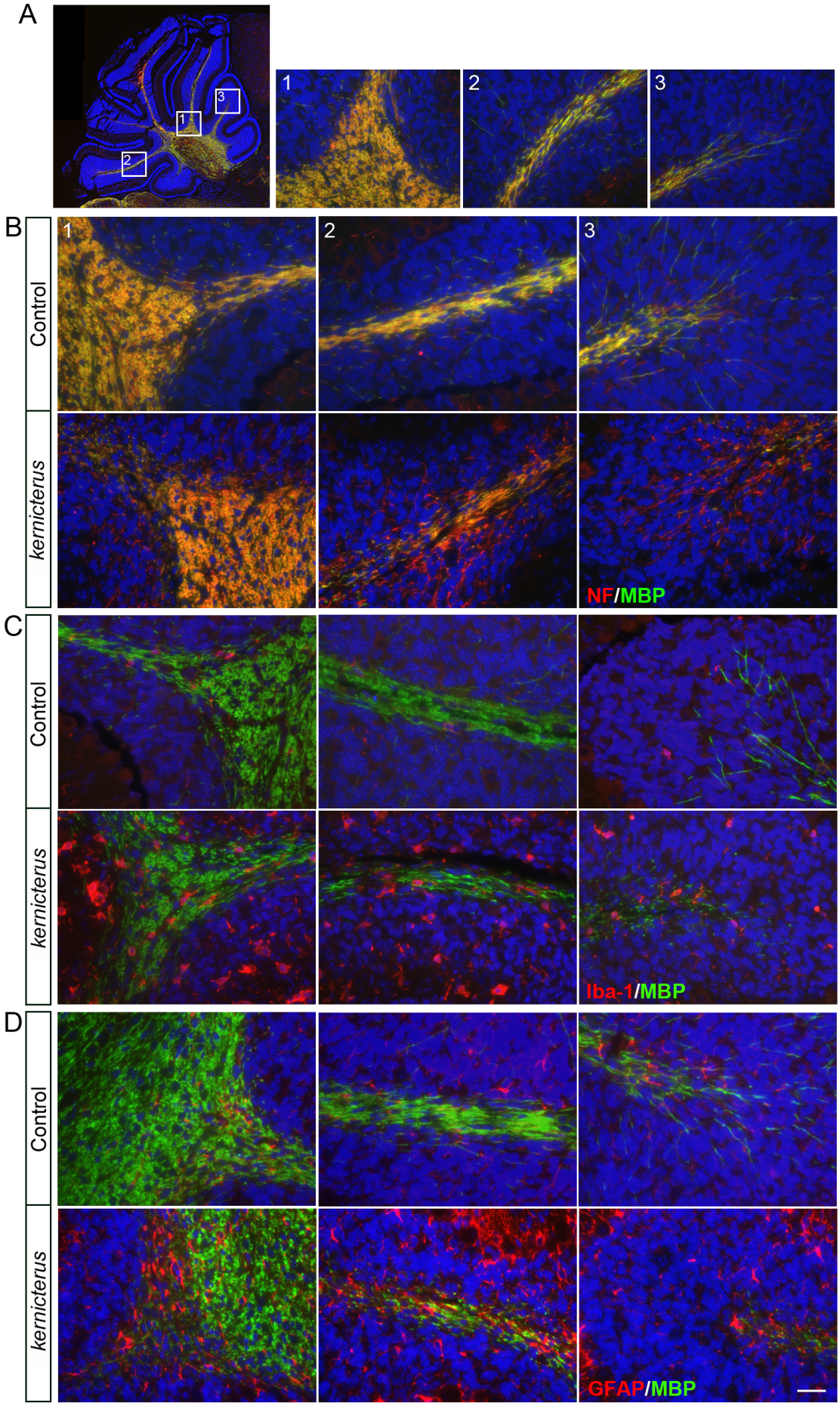

A
1
2
3
B
Control
kernicterus
NF/MBP
C
Control
kernicterus
Iba-1/MBP
D
Control
kernicterus
GFAP/MBP

Fig. V.4 – Myelination and glial reactivity changes in different areas of cerebellum white matter in the *kernicterus* mice. a Three different regions were specified, Region 1 (white matter nodes), Region 2 (middle of white matter tracts) and Region 3 (white matter terminals). Sections from control and *kernicterus* mice were immunolabelled to identify **b** neurons (neurofilament, red) and myelin basic protein (MBP, green), **c** microglia (ionized calcium binding adaptor molecule, Iba-1+ staining, red) and MBP (green) and **d** astrocytes (glial fibrillary acidic protein, GFAP+ staining, red) and MBP (green). Nuclei were counterstained with DAPI dye (blue). Representative images from the three white mater regions are shown. Scale bar represents 25 µm.

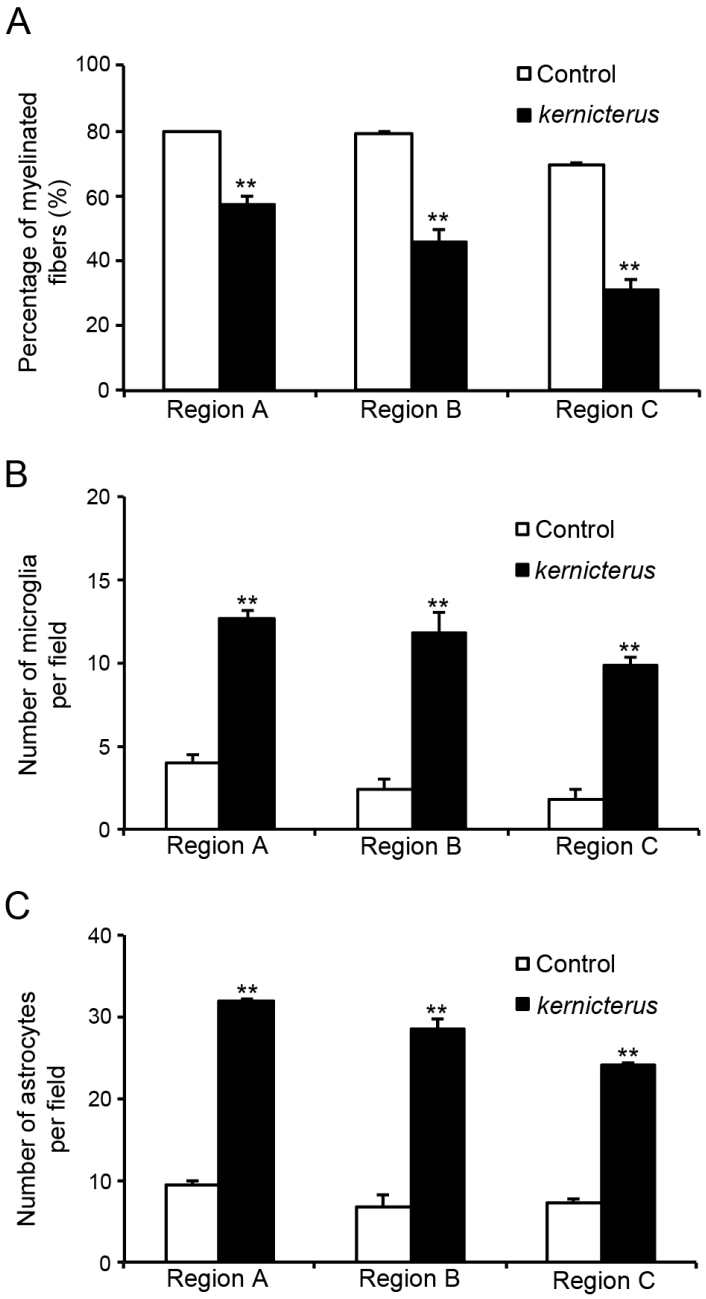


Fig. V.5 – Reduced myelinated fibers and increased microglia and astroglia burden in the *kernicterus* mice, different changes throughout white matter areas. Graph bars represent the quantification of percentage of myelinated fibers (**a**), the number of microglia (**b**) and astrocytes (**c**) per field in different white matter regions from control and *kernicterus* mice. Results are mean ± SEM from two control mice and three *kernicterus* mice performed in triplicate. ***P*<0.01 vs. respective control.

3.5. *Kernicterus* mice present cerebellum atrophy and a great reduction in Purkinje cell number

Besides changes observed in myelination and glial reactivity, it was already shown here that cerebellum volume is greatly reduced in *kernicterus* animals, a feature also observed in the Gunn rat model (Conlee and Shapiro 1997). As depicted in Figs. V.1 and V.6a, *kernicterus* mice, in relation to control ones, present a marked derangement of cerebellar lobules resulting in a great reduction in the area occupied by cerebellum when compared with total brain area (0.45±0.03 fold, $P<0.01$). This cerebellum atrophy in *kernicterus* mice can be due, to a massive reduction in the number of Purkinje cells (0.21±0.03 fold, $P<0.01$) mainly in the anterior lobes, and shrinkage of molecular and granular layers, as shown in Fig. V.6a. Moreover, when we looked more closely at Purkinje cells that remained in the *kernicterus* mice (Fig. V.6b), they presented an altered morphology with reduced number of branches suggesting that cerebellar neuronal circuitry may be markedly affected.

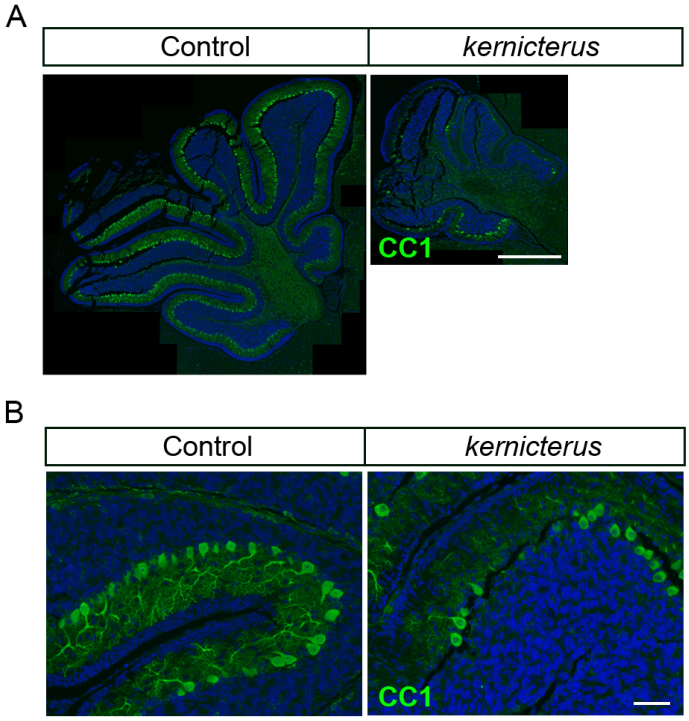


Fig. V.6 – *Kernicterus* mice present cerebellum atrophy, loss of Purkinje cells and less branching in the remaining cells. a 3 μm sections of each animal were immunolabelled with Adenomatus Polyposis Coli (CC1) to identify Purkinje cells and serial sagittal images were acquired from cerebellum of control and *kernicterus* mice. Each image represents a montage of 25-50 images at 10x magnification. Nuclei were counterstained with DAPI dye (blue). Scale bar represents 700 μm. **b** Representative images of Purkinje cells morphology in control and *kernicterus* mice. Scale bar represents 25 μm.

4. Discussion

The results demonstrate that this new animal model of severe hyperbilirubinemia/*kernicterus* present a marked cerebellar hypoplasia in parallel with a reduction of myelination and an increase in astrogliosis and microgliosis not only in the cerebellum but also in the pons and medulla oblongata after 7 postnatal days. In comparison with the Gunn rat model, these *kernicterus* mice present increased levels of circulating UCB within the first week of life without the need of any further treatment with an UCB to albumin displacer, being so considered a better model to mimic the human neonatal condition of hyperbilirubinemia.

We have recently demonstrated that UCB impairs oligodendrocyte differentiation and consequent myelination in the *in vitro* myelination model of dorsal-root ganglia neuron-oligodendrocyte co-cultures (Barateiro et al. 2012b) and in the *ex vivo* model of organotypic cerebellar slice cultures (Barateiro et al. 2012c). These observations are consistent with the previous reports showing a decrease in the density of myelinated fibers and a loss of axons in the cerebellum of a premature infant with *kernicterus* (Brito et al. 2012), and the white matter volume reduction and delay in hemispheric myelination observed in infants with severe UCB encephalopathy outcomes (Gkoltsiou et al. 2008). Consistently, here we show a marked impairment of myelination in the *kernicterus* mice. Indeed, it is easily observed the fragmentation of myelinated fibers within the cerebellum, medulla oblongata and pons, suggesting damaging of the myelin sheath. In accordance, studies perfomed in an experimental model of neonatal rabbit-induced *kernicterus* showed alterations of myelin sheat compactation (Chen et al. 1971), while in the Gunn model it was described changes in myelin, with myelin around vacuoles, bits of cytoplasm or other cytoplasmic debries (Jew et al. 1977), and a decreased expression of brain MBP (O'Callaghan and Miller 1985). Myelination deficits have also been reported in several other perinatal conditions including moderate perinatal systemic inflammation (Favrais et al. 2011), perinatal hypoxic-ischemia (Huang et al. 2009) and white matter injury in the premature baby (Buser et al. 2012). Since it is known that neuronal-oligodendrocyte cross-talk is crucial for a proper myelination (Lee and Fields 2009), myelination impairment observed in the *kernicterus* mice may be due in part to a reduced number of viable axons that cannot promote a proper oligodendrocyte differentiation and myelination. Nevertheless, the percentage of myelination in the remaining fibers was also affected in these animals corroborating the toxic role of UCB on oligodendrocyte maturation previously seen by us in the *in vitro* models. Indeed, as reported for white matter injury (Buser et al. 2012),

myelination failure may arise from inability of oligodendrocyte precursors to generate myelinating oligodendrocytes in order to repair the injury.

Here, we also show that *kernicterus* mice present an enhanced astrogliosis and microgliosis, corroborating our previous study in organotypic cerebellar slice cultures (Barateiro et al. 2012c). Here we showed an increased burden of astrocytes and microglia following UCB treatment mostly in the cerebellum, but also in pons and medulla oblongata. Interestingly, in the Gunn rat model it was only described astrocyte hypertrophy in the cerebellum with no hyperplasia (Mikoshiba et al. 1980), in parallel with microglia hyperplasia in the cerebellum (Keino et al. 1986) and activation within the hippocampus (Liaury et al. 2012). Hence, it seems that our new *kernicterus* animal model appears to present increased and more diffuse glia reactivity when compared to the Gunn rats. To this it may account the different genesis of the hyperbilirubinemia or even the different species reactivity.

It deserves to be noted that the increased astrogliosis and microgliosis in the *kernicterus* mice overlay with the brain areas that present myelin damage. This fact is even more notorious in the cerebellum images (Fig. V.4) where we can see the recruitment of both microglia and astrocytes to the surrounding white matter. It is known that upon myelin damage both astrocytes and microglia are rapidly activated and migrate to the site of injury (Petzold et al. 2002) with sustained astrocyte presence along myelination (Miron et al. 2010; Petzold et al. 2002), even during the perinatal period (Buser et al. 2012; Huang et al. 2009). Upon demyelination, microglia is responsible for clearing myelin debris to allow a proper remyelination (Olah et al. 2012). In addition, astrocytes and microglia produce growth factors that can facilitate oligodendrocyte survival, differentiation and ability to myelinate (Zhang et al. 2006; Stankoff et al. 2002; Olah et al. 2012), creating a favourable environment for repair. Indeed, we see an increased and acute number of microglia in the most affected areas of myelin deficits suggesting that these cells may be recruited to clear the damaged myelin.

On the other hand, if highly activated, microglia and astrocytes may release high amounts of pro-inflammatory mediators that are known toxicants for oligodendrocytes or their progenitors. Indeed, our recent study in organotypic cerebellar cultures showed that UCB promotes increased releases of glutamate and tumor necrosis factor (TNF)-α, known oligodendrocyte toxicants (Barateiro et al. 2012c). In accordance, it was recently reported that activated microglia following hypoxia produce excessive amounts of inflammatory cytokines including TNF-α and interleukin (IL)-1β along with glutamate, nitric oxide and reactive oxygen species, which collectively cause oligodendrocyte death and axonal degeneration. Moreover, the

authors also shown that activated microglia induced Purkinje neuronal death through TNF-α and IL-1β signalling (Kaur et al. 2012). Therefore, both myelination impairments and loss of Purkinje cells in the *kernicterus* model may be due to oligodendrocyte damage by these glial-derived inflammatory factors. In addition, it was recently reported that following white matter injury in the premature, astrogliosis extended into the site of injury and express elevated levels of hyaluronic acid that inhibits oligodendrocyte differentiation and consequent myelination into the astrogliotic lesion (Buser et al. 2012). Interestingly, our results show an increased astrogliosis in cerebellum central white matter tracts, regions that do not present the highest levels of myelination deficits, but are in the way of the most affected nerve fibers at white matter terminals. So, in the *kernicterus* model astrogliosis may be impairing the proper resolution of myelin damage and consequent remyelination.

Cerebellar hypoplasia was the more marked feature observed in the *kernicterus* mice. Indeed, our study reveals that increased circulating levels of UCB derange cerebellar lobules, with a reduction of the thickness of the molecular and granular layers, and a marked loss of Purkinje cells with reduced branching of the remaining ones, features that was also shown in the Gunn rat model (O'Callaghan and Miller 1985; Conlee and Shapiro 1997). The rodent cerebellar lobules mature postnatally, increasing over 20-fold from birth to 21 postnatal days. Interestingly, the cells within the ventral lobes, namely Purkinje cells, mature first than the ones in the anterior lobes (Altman 1969), what may play a role in the susceptibility to UCB. Here, we have observed an increased loss of Purkinje cells mostly in the anterior lobe indicating that the area that is under maturation may be more affected by UCB exposure than the one that is already mature. This fact may be corroborated by our earlier findings showing that immature neurons and astrocytes are more susceptible to UCB toxicity and cell death than mature ones (Falcão et al. 2006).

The cerebellum is a region of the brain that plays an important role in motor control, although not responsible for movement initiation, it contributes to coordination, precision, and accurate timing (Fine et al. 2002). Therefore, damage to cerebellum, as observed in the *kernicterus* model, may justify the abnormal motor control, movements and muscle tone observed in infants following severe neonatal hyperbilirrubinemia (Shapiro 2010). Interestingly, in the present model we also show alterations in several brain areas that are commonly affected in neonatal severe hyperbilirubinemia (Bhutani and Stevenson 2011), but not commonly described as injured in the Gunn rat model. Indeed, we also observed myelin deficits and gliosis in medulla oblongata and pons of *kernicterus* mice. Both medulla oblongata and pons are structures located on the brainstem, while medulla is the lower half of the brainstem, the pons is above the

medulla, below the midbrain and ventral to cerebellum. The medulla oblongata connects the higher levels of the brain to the spinal cord and has an important role in the control of nervous system autonomic functions, including the reflex centre of swallowing (Jean 1984). Interestingly, a history of past or present suckling and swallowing dysfunction is often described in children with *kernicterus* (Shapiro 2010), which may occur as a result of altered function of the medulla nerve cells as observed in our animal model. On the other hand, the pons white matter includes tracts that conduct signals from the cerebrum down to the cerebellum and medulla, and tracts that carry the sensory signals up into the thalamus. Interestingly, several cranial nerve nuclei are present in the pons, including the motor nucleus for the trigeminal nerve and the vestibulocochlear nuclei (Angeles Fernandez-Gil et al. 2010), responsible for eye movement and hearing, respectively. So, damage to these nuclei may be in the genesis of oculomotor impairments and auditory processing disturbance found in severe jaundice (Shapiro 2010).

Overall, our study reveals a new animal model of *kernicterus* showing anatomical and histological changes that may justify the clinical symptoms often detected in infants subjected to severe neonatal hyperbilirubinemia. Moreover, we have identified for the first time deficits in myelination and enhanced gliosis in the *kernicterus* mice, mechanisms that may be responsible for the neurological dysfunction described in moderate to severe hyperbilirubinemia, and may then be viewed as potential targets for new therapeutic strategies.

5. References

Altman J. 1969. Autoradiographic and histological studies of postnatal neurogenesis. 3. Dating the time of production and onset of differentiation of cerebellar microneurons in rats. J Comp Neurol 136(3):269-93.

Angeles Fernandez-Gil M, Palacios-Bote R, Leo-Barahona M, Mora-Encinas JP. 2010. Anatomy of the brainstem: a gaze into the stem of life. Semin Ultrasound CT MR 31(3):196-219.

Barateiro A, Vaz AR, Silva SL, Fernandes A, Brites D. 2012a. ER Stress, Mitochondrial Dysfunction and Calpain/JNK Activation are Involved in Oligodendrocyte Precursor Cell Death by Unconjugated Bilirubin. Neuromolecular medicine, [Epub ahead of print].

Barateiro A, Miron VE, Santos SD, Relvas JB, Fernandes A, ffrench-Constant C, Brites D. 2012b. Arrest of oligodendrocyte differentiation and axonal myelination in an in vitro modelo f hyperbilirubinemia. Submitted.

Barateiro A, Domingues HS, Fernandes A, Relvas JB, Brites D. 2012c. Cerebellum slice culture model mimicking hyperbilirubinemia: changes in oligodendrocyte maturation, myelinogenesis and glial reactivity. Submitted.

Bhutani VK, Stevenson DK. 2011. The need for technologies to prevent bilirubin-induced neurologic dysfunction syndrome. Seminars in perinatology 35(3):97-100.

Brito MA, Vaz AR, Silva SL, Falcão AS, Fernandes A, Silva RFM, Brites D. 2010. N-methyl-aspartate receptor and neuronal nitric oxide synthase activation mediate bilirubin-induced neurotoxicity. Molecular medicine 16(9-10):372-80.

Brito MA, Zurolo E, Pereira P, Barroso C, Aronica E, Brites D. 2012. Cerebellar axon/myelin loss, angiogenic sprouting, and neuronal increase of vascular endothelial growth factor in a preterm infant with kernicterus. J Child Neurol 27(5):615-24.

Buser JR, Maire J, Riddle A, Gong X, Nguyen T, Nelson K, Luo NL, Ren J, Struve J, Sherman LS and others. 2012. Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. Ann Neurol 71(1):93-109.

Chen HC, Wang CH, Tsan KW, Chen YC. 1971. An electron microscopic and radioautographic study on experimental kernicterus. II. Bilirubin movement within neurons and release of waste products via astroglia. The American journal of pathology 64(1):45-66.

Cohen RS, Wong RJ, Stevenson DK. 2010. Understanding neonatal jaundice: a perspective on causation. Pediatrics and neonatology 51(3):143-8.

Conlee JW, Shapiro SM. 1997. Development of cerebellar hypoplasia in jaundiced Gunn rats: a quantitative light microscopic analysis. Acta Neuropathol 93(5):450-60.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2005. Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. Neurobiology of disease 20(2):199-206.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta neuropathologica 112(1):95-105.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta Neuropathol 112(1):95-105.

Favrais G, van de Looij Y, Fleiss B, Ramanantsoa N, Bonnin P, Stoltenburg-Didinger G, Lacaud A, Saliba E, Dammann O, Gallego J and others. 2011. Systemic inflammation disrupts the developmental program of white matter. Ann Neurol 70(4):550-65.

Fernandes A, Falcão AS, Silva RFM, Gordo AC, Gama MJ, Brito MA, Brites D. 2006. Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. Journal of neurochemistry 96(6):1667-79.

Fernandes A, Silva RFM, Falcão AS, Brito MA, Brites D. 2004. Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. Journal of neuroimmunology 153(1-2):64-75.

Fine EJ, Ionita CC, Lohr L. 2002. The history of the development of the cerebellar examination. Semin Neurol 22(4):375-84.

Gazzin S, Strazielle N, Schmitt C, Fevre-Montange M, Ostrow JD, Tiribelli C, Ghersi-Egea JF. 2008. Differential expression of the multidrug resistance-related proteins ABCb1 and ABCc1 between blood-brain interfaces. J Comp Neurol 510(5):497-507.

Gkoltsiou K, Tzoufi M, Counsell S, Rutherford M, Cowan F. 2008. Serial brain MRI and ultrasound findings: relation to gestational age, bilirubin level, neonatal neurologic status and neurodevelopmental outcome in infants at risk of kernicterus. Early Hum Dev 84(12):829-38.

Gordo AC, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Unconjugated bilirubin activates and damages microglia. Journal of neuroscience research 84(1):194-201.

Grojean S, Lievre V, Koziel V, Vert P, Daval JL. 2001. Bilirubin exerts additional toxic effects in hypoxic cultured neurons from the developing rat brain by the recruitment of glutamate neurotoxicity. Pediatric research 49(4):507-13.

Gunn CK. 1944. Hereditary Acholuric Jaundice in the Rat. Canadian Medical Association journal 50(3):230-7.

Hanko E, Hansen TW, Almaas R, Lindstad J, Rootwelt T. 2005. Bilirubin induces apoptosis and necrosis in human NT2-N neurons. Pediatric research 57(2):179-84.

Huang Z, Liu J, Cheung PY, Chen C. 2009. Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury. Brain Res 1301:100-9.

Jean A. 1984. Brainstem organization of the swallowing network. Brain Behav Evol 25(2-3):109-16.

Jew JY, Williams TH. 1977. Ultrastructural aspects of bilirubin encephalopathy in cochlear nuclei of the Gunn rat. Journal of anatomy 124(Pt 3):599-614.

Johnson L, Bhutani VK. 2011. The clinical syndrome of bilirubin-induced neurologic dysfunction. Semin Perinatol 35(3):101-13.

Kaur C, Rathnasamy G, Ling EA. 2012. Roles of Activated Microglia in Hypoxia Induced Neuroinflammation in the Developing Brain and the Retina. J Neuroimmune Pharmacol.

Keino H, Aoki E, Kashiwamata S. 1986. Postnatal changes of the number and lobular distribution of acid phosphatase positive and lipid granule-containing cells in the cerebellum of hyperbilirubinemic Gunn rats. Neurosci Res 3(3):183-95.

Lee PR, Fields RD. 2009. Regulation of myelin genes implicated in psychiatric disorders by functional activity in axons. Front Neuroanat 3:4.

Liaury K, Miyaoka T, Tsumori T, Furuya M, Wake R, Ieda M, Tsuchie K, Taki M, Ishihara K, Tanra AJ and others. 2012. Morphological features of microglial cells in the hippocampal dentate gyrus of Gunn rat: a possible schizophrenia animal model. J Neuroinflammation 9:56.

McDonald JW, Shapiro SM, Silverstein FS, Johnston MV. 1998. Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. Experimental neurology 150(1):21-9.

Mikoshiba K, Kohsaka S, Takamatsu K, Tsukada Y. 1980. Cerebellar hypoplasia in the Gunn rat with hereditary hyperbilirubinemia: immunohistochemical and neurochemical studies. J Neurochem 35(6):1309-18.

Miron VE, Ludwin SK, Darlington PJ, Jarjour AA, Soliven B, Kennedy TE, Antel JP. 2010. Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. Am J Pathol 176(6):2682-94.

O'Callaghan JP, Miller DB. 1985. Cerebellar hypoplasia in the Gunn rat is associated with quantitative changes in neurotypic and gliotypic proteins. J Pharmacol Exp Ther 234(2):522-33.

Olah M, Amor S, Brouwer N, Vinet J, Eggen B, Biber K, Boddeke HW. 2012. Identification of a microglia phenotype supportive of remyelination. Glia 60(2):306-21.

Petzold A, Eikelenboom MJ, Gveric D, Keir G, Chapman M, Lazeron RH, Cuzner ML, Polman CH, Uitdehaag BM, Thompson EJ and others. 2002. Markers for different glial cell responses in multiple sclerosis: clinical and pathological correlations. Brain 125(Pt 7):1462-73.

Schutta HS, Johnson L. 1969. Clinical signs and morphologic abnormalities in Gunn rats treated with sulfadimethoxine. The Journal of pediatrics 75(6):1070-9.

Shapiro SM. 2010. Chronic bilirubin encephalopathy: diagnosis and outcome. Semin Fetal Neonatal Med 15(3):157-63.

Silva SL, Osorio C, Vaz AR, Barateiro A, Falcão AS, Silva RFM, Brites D. 2011. Dynamics of neuron-glia interplay upon exposure to unconjugated bilirubin. Journal of neurochemistry 117(3):412-24.

Silva SL, Vaz AR, Barateiro A, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2010. Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. Neurobiology of disease 40(3):663-75.

Silva SL, Vaz AR, Diogenes MJ, van Rooijen N, Sebastiao AM, Fernandes A, Silva RFM, Brites D. 2012. Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia, and prevented by glycoursodeoxycholic acid and interleukin-10. Neuropharmacology 62(7):2398-408.

Stevenson DK, Dennery PA, Hintz SR. 2001. Understanding newborn jaundice. Journal of perinatology : official journal of the California Perinatal Association 21 Suppl 1:S21-4; discussion S35-9.

Sturrock RR. 1980. Myelination of the mouse corpus callosum. Neuropathol Appl Neurobiol 6(6):415-20.

Sturrock RR, Jew JY. 1978. A quantitative histological study of changes in neurons and glia in the Gunn rat brain. Neuropathology and applied neurobiology 4(3):209-23.

Vaz AR, Delgado-Esteban M, Brito MA, Bolanos JP, Brites D, Almeida A. 2010. Bilirubin selectively inhibits cytochrome c oxidase activity and induces apoptosis in immature cortical neurons: assessment of the protective effects of glycoursodeoxycholic acid. Journal of neurochemistry 112(1):56-65.

Zhang Y, Taveggia C, Melendez-Vasquez C, Einheber S, Raine CS, Salzer JL, Brosnan CF, John GR. 2006. Interleukin-11 potentiates oligodendrocyte survival and maturation, and myelin formation. J Neurosci 26(47):12174-85.

Chapter VI

FINAL CONSIDERATIONS

1. Concluding remarks and perspectives

The main goal of this thesis was to explore if neonatal hyperbilirubinemia and associated conditions, such as inflammation, impairs oligodendrocyte (OL) development and consequently the myelination process, that would explain the changes in myelination observed in previous studies performed in autopsy cases of severe hyperbilirubinemia (Brito et al. 2012; Gkoltsiou et al. 2008).

Although, several *in vitro* studies have demonstrated that unconjugated bilirubin (UCB) is cytotoxic to nerve cells, including neurons, astrocytes and microglia (Brites 2012), only one study showed that high levels of UCB implicate a decrease in viability of one type of cells from OL lineage, the mature OL (Genc et al. 2003). Several mechanisms of UCB neurotoxicity were ultimately dissected as the pathways leading to an inflammatory response in astrocytes and microglia, that our group showed to be mediated through the activation of mitogen-protein kinases and nuclear factor-kappaB signalling cascades (Fernandes et al. 2006; Fernandes et al. 2004; Gordo et al. 2006; Silva et al. 2011; Silva et al. 2010).

However, in what concerns myelination only a few studies have demonstrated the deleterious effects of UCB in this process. Several years ago, studies performed in tissue cultures of rat cerebellum showed that UCB has a deleterious effect in myelination (Silberberg and Schutta 1967), while others pointed that UCB binds to myelin basic protein (MBP) *in vitro* (Gurba and Zand 1974), and that high levels are present *in vivo* in the myelin fraction after UCB injection (Hansen et al. 2001). More recent studies revealed that infants with severe hyperbilirubinemia present reduction of white matter volume and delay in hemispheric myelination (Gkoltsiou et al. 2008), while in a premature case with *kernicterus* it was identified a decreased density of myelinated fibers in cerebellum (Brito et al. 2012). Therefore, it is important to better understand the response of OL at different stages of maturation to UCB and the mechanisms involved in UCB-induced toxicity.

First of all we decided to evaluate the effect of UCB in oligodendrocyte progenitor cell (OPC) primary cultures (**Chapter II**). These cells are known to be the most vulnerable cells from OL lineage and were never evaluated for the mechanisms of UCB-neurotoxicity as it was done to both immature astrocytes and neurons, showing its increased vulnerability when compared to differentiated ones (Falcão et al. 2005; Falcão et al. 2006). So, the study was pioneer in accessing changes in OPC viability and the mechanisms involved in UCB-induced toxicity. Cells were kept in culture with PDGF and FGF, to promote proliferation and block differentiation. UCB has shown to induce an increase in OPC death by apoptosis and necrosis-like cell death, involving

early signals of ER stress and mitochondrial dysfunction, not involving any inflammatory response or glutamate release. UCB promoted a rapid and transient upregulation of glucose-regulated protein (GRP) 78, inositol-requiring enzyme (IRE)-1α and activating transcription factor (ATF)-6, calling to the accumulation of unfolded proteins that encompass the unfolded protein response pathway. Endoplasmic reticulum (ER) stress led to caspase-2 and c-Jun *N*-terminal kinase (JNK) activation, and hypothetically to mitochondrial dysfunction. In fact, it caused a loss of mitochondria potential, production of reactive oxygen species (ROS), as well as activation of caspase-9 and -3. The generation of ROS may be responsible for the second cycle of GRP78, IRE-1α, caspase-2 and JNK activation observed at later times. ER stress and mitochondrial dysfunction, as the major organelles involved in Ca^{2+} homeostasis, can then be considered in the origin of UCB-induced activation of calpains, thus suggesting an increase in the intracellular concentration of Ca^{2+}. By using specific caspases, calpains and oxidative stress inhibitors, we were able to show that activation of all these pathways is required for UCB-induced OPC death. ER stress by UCB may thus lead to caspase-2 activation and mitochondrial dysfunction, with consequent caspase-9 activation, as observed in myeloma cells (Gu et al. 2008). In fact, UCB was shown to impair mitochondrial respiratory chain in immature neurons (Vaz et al. 2010). Here, we propose that ER stress also induces JNK activation, since Tiberio and collaborators (2012) have demonstrated that oxidative and ER stress, followed by JNK activation, mediate apoptosis. However, activation of JNK activation may additionally result from perturbations in Ca^{2+} homeostasis due to ER stress, triggering further release of Ca^{2+} and JNK mediation of the ensuing ER-mitochondria *cross-talk* (Verma and Datta 2012). Besides, mitochondria-dependent death pathway mediated by calpain was already demonstrated in OL (Fu et al. 2009; Sánchez-Gómez et al. 2011) and our data really suggest a role for UCB at this level.

Overall, the results achieved in this chapter indicate that UCB compromise oligodendrogenesis[1].

[1] Barateiro A, Vaz AR, Silva SL, Fernandes A, Brites D. 2012. ER Stress, Mitochondrial Dysfunction and Calpain/JNK Activation are Involved in Oligodendrocyte Precursor Cell Death by Unconjugated Bilirubin. Neuromolecular medicine, [Epub ahead of print].

Since UCB activates several death pathways in OPC, we next evaluated the alterations produced by UCB in OL maturation and their ability to myelinate (**Chapter III**). We assessed not only the alterations produced by UCB over the different stages of OL maturation, but also at the morphological maturation process, once OL differentiation is a fundamental step for the beginning of myelination. As well, the increased morphological complexity and the ability to elaborate membranes are important features of terminal OL differentiation. In this context, we demonstrated that UCB exposure at different stages of OL development had different impacts in their differentiation and maturation. If exposure takes place prior to the onset of differentiation, UCB determines a delay in OL differentiation by increasing the number of OPC (NG2+ staining) and decreasing that of mature OL (MBP+ staining). This may result from the decrease in Olig1 and increase in Olig2 mRNA levels detected after UCB treatment. Further examination at the mature OL revealed that UCB added prior or in the presence of differentiation factors, delays OL morphological maturation, by impairment of process extension, reduction of the membrane surface and diameter of MBP+ cells. These findings were corroborated by the marked reduction in active guanosine triphosphate (GTP)-bound Rac1 fraction caused by either treatment, suggesting that cytoskeleton may be also a target for UCB.

To myelinate properly, OL should be in contact with neuronal axons, so we then used myelinating dorsal root ganglia (DRG) neuron-OL co-cultures. While exposure to UCB prior to the beginning of myelination delayed OPC differentiation into MBP+ OL, and their morphological maturation, exposure during myelination evidenced only a delay in OL morphological maturation. This different response may be explained taking in consideration that if addition of UCB is performed during myelination, the OPC differentiation has already started and, therefore, UCB can only impair the last steps of morphological maturation. However, we demonstrate here that both treatments cause myelination defects, as evidenced by the decrease in both the number of myelinated internodes per OL and the internode length. So, both OL maturation and myelination are affected by UCB. Similar results were observed for cytokines (Chew et al. 2005; Favrais et al. 2011; Mann et al. 2008), diffuse white matter injury (Buser et al. 2012) and for chronic hypoxia-ischemia-induced periventricular white matter injury (Segovia et al. 2008). Interestingly, these alterations in OL maturation stage can be due to changes in Olig1 and Olig2 expression, since Olig1 is essential for myelinogenesis and has higher expression during membranes maintenance, while Olig2 is mostly expressed in more immature cells (Niu et al. 2012; Othman et al. 2011; Xin et al. 2005). Moreover, the reduction in active Rac1 by UCB may be another determinant of the OL morphological defects as already demonstrated by depletion of active Rac1 (Wang et

al. 2009), since active Rac1 is important for myelin sheath formation (Czopka et al. 2009). However, we must not exclude the contribution of other alterations in these impaired maturation and myelination events. So it will be important in the future to explore additional features that may be induced by UCB such as those on electrical signals and on additional axon cytoskeleton proteins, given their important role in OL survival, differentiation and myelination initiation (Fressinaud et al. 2012; Gary et al. 2012).

In here, the data further suggest that UCB causes hypomyelination by impairing OPC differentiation if present during the proliferation phase and by delaying premyelinating OL maturation and myelination programs[2].

Chapter IV was envisaged because the myelination process is not only regulated by OL and neurons, once astrocytes and microglia also take an important part. Thus, we used an *ex vivo* organotypic cerebellar slice model in order to have communication between all cells of the cerebellum, one of the most affected brain regions in bilirubin-induced neurological disfunction (BIND). In this new model we still observed that UCB triggers an increase in immature OL and a decrease in the number of myelinated fibers, with consequent decrease in Olig1 and increase in Olig2 mRNA expression. Increased astrocytosis and microgliosis evidenced to be directly related with the time of exposure, as it was the release of tumor necrosis factor (TNF)-α and glutamate, previously demonstrated in pure astrocyte and microglia cultures after exposure to UCB (Fernandes et al. 2004; Silva et al. 2010). Together, TNF-α and glutamate may have deleterious effects in myelination. In fact, these results are in line with a very recent study demonstrating that TNF-α exposure lead to myelination impairment (Pang et al. 2012). In addition, increased concentrations of glutamate may be, in part, responsible for the delay of OL lineage progression (Gallo et al. 1996; Yuan et al. 1998) and contribute to an increase in TNF-α release by microglia. Thus, it will be important to assess if TNF-α signalling blockers (infliximab) or silencing of the TNF receptor 1 (Fernandes et al. 2011) will prevent the arrest in OL differentiation and the myelination impairment. Among other interesting issues that can be addressed is to understand whether these UCB effects are predominantly due to microglia activation. For that we can deplete cerebellar slice cultures from microglia, by using clodronate liposomes, prior to UCB treatment. Indeed, a previous study using hippocampal slice

[2] Barateiro A, Miron VE, Santos SD, Relvas JB, Fernandes A, ffrench-Constant C, Brites D. 2012b. Arrest of oligodendrocyte differentiation and axonal myelination in an in vitro modelo f hyperbilirubinemia. Submitted.

cultures showed a reduced cell death and nitric oxide release in microglia depleted slices treated with UCB (Silva et al. 2012).

This chapter, using a closer *in vivo* model, outlined that besides the reduction in the percentage of myelinated fibers and the increase in the OPC number in the cerebellar slice cultures, UCB concomitantly causes astrocytosis and microgliosis, which may additionally have detrimental roles, mainly in chronic hyperbilirubinemia[3].

Finally, in the last experimental **Chapter V** we have obtained convincing data that the molecular mechanisms of myelinogenesis impairment by UCB also occur in a new model of *kernicterus* that is being produced and characterized by the Department of Pharmacology at the University of California San Diego by Robert Tukey. Changes produced by hyperbilirubinemia in these mice caused modifications of brain morphology, with a great atrophy of the cerebellum. Decreased myelinated fibers were observed in the brain regions compromised by UCB encephalopathy, including the medulla oblongata, pons and the cerebellum, where it was noticed an increased number of astrocytes and microglia. By exploring the cerebellum histopathology this novel study evidenced that myelination impairment and microgliosis occur mostly in white matter terminals and in the middle of white matter tracts, the place where we also observed the presence of astrocytosis[4]. How far these insults can be reversed is a question with no answer yet. Astrocytes present along the myelination process (Miron et al. 2010; Petzold et al. 2002) and microglia that perform myelin debris clearance to allow remyelination (Olah et al. 2012), are cells that if functionally compromised or overactivated, as evidenced in the previous Chapter, induce OL death, axonal degeneration and Purkinje cell death (Kaur et al. 2012). Regarding cerebellum abnormalities observed in this model, they could be responsible for the abnormal motor control, movements and muscle tone that *kernicterus* infants present (Shapiro 2010). Curiously, changes that we observed in medulla oblongata and pons, usually affected in *kernicterus* infants (Bhutani and Stevenson 2011), are not reproduced in the most used jaundiced model, the Gunn rat, attesting that this new model may be better to evaluate the mechanisms of UCB-induced neurotoxicity, as well as the sequelae that may be produced. In fact, medulla oblongata alterations can be related with the suckling and swallowing dysfunction observed in *kernicterus* cases (Shapiro 2010), while alterations in pons may be responsible for the oculomotor impairments and

[3] Barateiro A, Domingues HS, Fernandes A, Relvas JB, Brites D. 2012. Cerebellum slice culture model mimicking hyperbilirubinemia: changes in oligodendrocyte maturation, myelinogenesis and glial reactivity. Submitted.

[4] Barateiro A, Fernandes A, Domingues HS, Relvas JB, Chen S, Tukey R, Brites D. 2012. Reduced myelination and increased glia reactivity in a hyperbilirubinemic mouse model. Submitted.

auditory processing disturbance found in these infants (Shapiro 2010). Studies in the future using these animals are promising in allowing the connection between this common condition in the neonatal period life and the vulnerability to aging and to mental disorders in later life.

Alltogether, the data presented in this thesis, and schematically represented in Figure VI.1, indicate that deficits in myelinogenesis should be considered among the neurotoxic mechanisms of BIND and provide some of the inter- and intracellular pathways additionally implicated in BIND, and not explored until now. In fact, increased concentrations of UCB have harmful effects in OL viability, maturation and myelination. Moreover, these findings can explain the myelination impairment in cases of severe hyperbilirubinemia and provide new insights for the development of new therapeutic strategies to prevent UCB-induced alterations in myelination, and set the basis for future investigations.

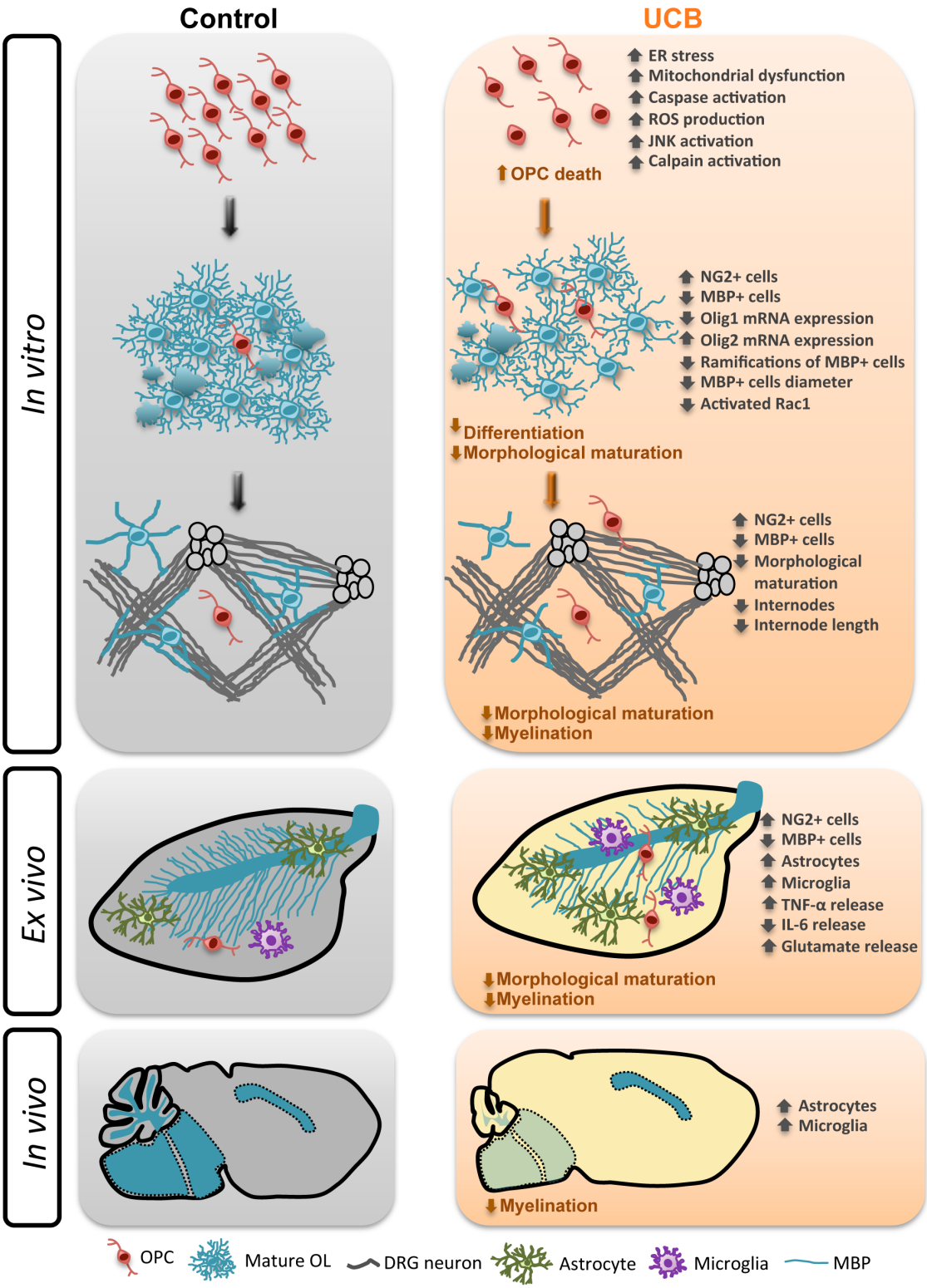


Fig. VI. 1 – Schematic representation of the major findings achieved in the present work. Studies *in vitro* performed in oligodendrocyte precursor cell (OPC) primary cultures, in oligodendrocyte maturation model and in dorsal root ganglia (DRG) neuron-oligodencrocyte (OL) co-culture, showed that unconjugated bilirubin leads to OPC death, to decreased differentiation and decreased myelination, respectively. In addition, *ex vivo* studies developed in cerebellar slice cultures showed that UCB exposure is able to induce a decrease in OL morphological maturation and myelination, probably due to an increase in astrocyte and microglia activation. Finally, *in vivo* studies in an animal model of *kernicterus* showed that these mice have marked cerebellum atrophy and decreased myelination in several brain regions, such as cerebellum, pons and medulla oblongata, with no significative changes in corpus callosum. MBP, myelin basic protein.

2. References

Bhutani VK, Stevenson DK. 2011. The need for technologies to prevent bilirubin-induced neurologic dysfunction syndrome. Seminars in perinatology 35(3):97-100.

Brites D. 2012. The evolving landscape of neurotoxicity by unconjugated bilirubin: role of glial cells and inflammation. Frontiers in pharmacology 3:88.

Brito MA, Zurolo E, Pereira P, Barroso C, Aronica E, Brites D. 2012. Cerebellar axon/myelin loss, angiogenic sprouting, and neuronal increase of vascular endothelial growth factor in a preterm infant with kernicterus. Journal of child neurology 27(5):615-24.

Buser JR, Maire J, Riddle A, Gong X, Nguyen T, Nelson K, Luo NL, Ren J, Struve J, Sherman LS and others. 2012. Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. Ann Neurol 71(1):93-109.

Chew LJ, King WC, Kennedy A, Gallo V. 2005. Interferon-gamma inhibits cell cycle exit in differentiating oligodendrocyte progenitor cells. Glia 52(2):127-43.

Czopka T, Von Holst A, Schmidt G, Ffrench-Constant C, Faissner A. 2009. Tenascin C and tenascin R similarly prevent the formation of myelin membranes in a RhoA-dependent manner, but antagonistically regulate the expression of myelin basic protein via a separate pathway. Glia 57(16):1790-801.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2005. Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. Neurobiology of disease 20(2):199-206.

Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta neuropathologica 112(1):95-105.

Favrais G, van de Looij Y, Fleiss B, Ramanantsoa N, Bonnin P, Stoltenburg-Didinger G, Lacaud A, Saliba E, Dammann O, Gallego J and others. 2011. Systemic inflammation disrupts the developmental program of white matter. Ann Neurol 70(4):550-65.

Fernandes A, Barateiro A, Falcão AS, Silva SL, Vaz AR, Brito MA, Silva RFM, Brites D. 2011. Astrocyte reactivity to unconjugated bilirubin requires TNF-alpha and IL-1beta receptor signaling pathways. Glia 59(1):14-25.

Fernandes A, Falcão AS, Silva RFm, Gordo AC, Gama MJ, Brito MA, Brites D. 2006. Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. Journal of neurochemistry 96(6):1667-79.

Fernandes A, Silva RFM, Falcão AS, Brito MA, Brites D. 2004. Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. Journal of neuroimmunology 153(1-2):64-75.

Fressinaud C, Berges R, Eyer J. 2012. Axon cytoskeleton proteins specifically modulate oligodendrocyte growth and differentiation in vitro. Neurochemistry international 60(1):78-90.

Fu Y, Sun W, Shi Y, Shi R, Cheng JX. 2009. Glutamate excitotoxicity inflicts paranodal myelin splitting and retraction. PloS one 4(8):e6705.

Gallo V, Zhou JM, McBain CJ, Wright P, Knutson PL, Armstrong RC. 1996. Oligodendrocyte progenitor cell proliferation and lineage progression are regulated by glutamate receptor-mediated K+ channel block. The Journal of neuroscience : the official journal of the Society for Neuroscience 16(8):2659-70.

Gary DS, Malone M, Capestany P, Houdayer T, McDonald JW. 2012. Electrical stimulation promotes the survival of oligodendrocytes in mixed cortical cultures. Journal of neuroscience research 90(1):72-83.

Genc S, Genc K, Kumral A, Baskin H, Ozkan H. 2003. Bilirubin is cytotoxic to rat oligodendrocytes in vitro. Brain research 985(2):135-41.

Gkoltsiou K, Tzoufi M, Counsell S, Rutherford M, Cowan F. 2008. Serial brain MRI and ultrasound findings: relation to gestational age, bilirubin level, neonatal neurologic status and neurodevelopmental outcome in infants at risk of kernicterus. Early human development 84(12):829-38.

Gordo AC, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2006. Unconjugated bilirubin activates and damages microglia. Journal of neuroscience research 84(1):194-201.

Gu H, Chen X, Gao G, Dong H. 2008. Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. Molecular cancer therapeutics 7(8):2298-307.

Gurba PE, Zand R. 1974. Bilirubin binding to myelin basic protein, histones and its inhibition in vitro of cerebellar protein synthesis. Biochemical and biophysical research communications 58(4):1142-7.

Hansen T, Tommarello S, Allen J. 2001. Subcellular localization of bilirubin in rat brain after in vivo i.v. administration of [3H]bilirubin. Pediatric research 49(2):203-7.

Kaur C, Rathnasamy G, Ling EA. 2012. Roles of Activated Microglia in Hypoxia Induced Neuroinflammation in the Developing Brain and the Retina. J Neuroimmune Pharmacol.

Mann SA, Versmold B, Marx R, Stahlhofen S, Dietzel ID, Heumann R, Berger R. 2008. Corticosteroids reverse cytokine-induced block of survival and differentiation of oligodendrocyte progenitor cells from rats. Journal of neuroinflammation 5:39.

Miron VE, Ludwin SK, Darlington PJ, Jarjour AA, Soliven B, Kennedy TE, Antel JP. 2010. Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. Am J Pathol 176(6):2682-94.

Niu J, Mei F, Wang L, Liu S, Tian Y, Mo W, Li H, Lu QR, Xiao L. 2012. Phosphorylated olig1 localizes to the cytosol of oligodendrocytes and promotes membrane expansion and maturation. Glia.

Olah M, Amor S, Brouwer N, Vinet J, Eggen B, Biber K, Boddeke HW. 2012. Identification of a microglia phenotype supportive of remyelination. Glia 60(2):306-21.

Othman A, Frim DM, Polak P, Vujicic S, Arnason BG, Boullerne AI. 2011. Olig1 is expressed in human oligodendrocytes during maturation and regeneration. Glia 59(6):914-26.

Pang Y, Zheng B, Kimberly SL, Cai Z, Rhodes PG, Lin RC. 2012. Neuron-oligodendrocyte myelination co-culture derived from embryonic rat spinal cord and cerebral cortex. Brain and behavior 2(1):53-67.

Petzold A, Eikelenboom MJ, Gveric D, Keir G, Chapman M, Lazeron RH, Cuzner ML, Polman CH, Uitdehaag BM, Thompson EJ and others. 2002. Markers for different glial cell responses in multiple sclerosis: clinical and pathological correlations. Brain 125(Pt 7):1462-73.

Sánchez-Gómez MV, Alberdi E, Perez-Navarro E, Alberch J, Matute C. 2011. Bax and calpain mediate excitotoxic oligodendrocyte death induced by activation of both AMPA and kainate receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 31(8):2996-3006.

Segovia KN, McClure M, Moravec M, Luo NL, Wan Y, Gong X, Riddle A, Craig A, Struve J, Sherman LS and others. 2008. Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. Annals of neurology 63(4):520-30.

Shapiro SM. 2010. Chronic bilirubin encephalopathy: diagnosis and outcome. Semin Fetal Neonatal Med 15(3):157-63.

Silberberg DH, Schutta HS. 1967. The effects of unconjugated bilirubin and related pigments on cultures of rat cerebellum. Journal of neuropathology and experimental neurology 26(4):572-83.

Silva SL, Osorio C, Vaz AR, Barateiro A, Falcão AS, Silva RFM, Brites D. 2011. Dynamics of neuron-glia interplay upon exposure to unconjugated bilirubin. Journal of neurochemistry 117(3):412-24.

Silva SL, Vaz AR, Barateiro A, Falcão AS, Fernandes A, Brito MA, Silva RFM, Brites D. 2010. Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. Neurobiology of disease 40(3):663-75.

Silva SL, Vaz AR, Diogenes MJ, van Rooijen N, Sebastiao AM, Fernandes A, Silva RFM, Brites D. 2012. Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia, and prevented by glycoursodeoxycholic acid and interleukin-10. Neuropharmacology 62(7):2398-408.

Tiberio P, Cavadini E, Callari M, Daidone MG, Appierto V. 2012. AF1q: A Novel Mediator of Basal and 4-HPR-Induced Apoptosis in Ovarian Cancer Cells. PloS one 7(6):e39968.

Vaz AR, Delgado-Esteban M, Brito MA, Bolanos JP, Brites D, Almeida A. 2010. Bilirubin selectively inhibits cytochrome c oxidase activity and induces apoptosis in immature cortical neurons: assessment of the protective effects of glycoursodeoxycholic acid. Journal of neurochemistry 112(1):56-65.

Verma G, Datta M. 2012. The critical role of JNK in the ER-mitochondrial crosstalk during apoptotic cell death. Journal of cellular physiology 227(5):1791-5.

Wang PS, Wang J, Xiao ZC, Pallen CJ. 2009. Protein-tyrosine phosphatase alpha acts as an upstream regulator of Fyn signaling to promote oligodendrocyte differentiation and myelination. J Biol Chem 284(48):33692-702.

Xin M, Yue T, Ma Z, Wu FF, Gow A, Lu QR. 2005. Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 25(6):1354-65.

Yuan X, Eisen AM, McBain CJ, Gallo V. 1998. A role for glutamate and its receptors in the regulation of oligodendrocyte development in cerebellar tissue slices. Development 125(15):2901-14.