UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

NEURONAL CYTOSKELETAL DYNAMIC MODIFICATION INDUCED BY UNCONJUGATED BILIRUBIN

Eduarda Cristina Duarte de Magalhães Coutinho

Mestrado em Biologia Molecular Humana

2009

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

NEURONAL CYTOSKELETAL DYNAMIC MODIFICATION INDUCED BY UNCONJUGATED BILIRUBIN

Dissertação orientada por:

Dora Maria Tuna de Oliveira Brites, PhD , Faculdade de Farmácia, dbrites@ff.ul.pt

Rui Manuel dos Santos Malhó, PhD, Faculdade de Ciências, rmmalho@fc.ul.pt

Eduarda Cristina Duarte de Magalhães Coutinho

Mestrado em Biologia Molecular Humana

2009

Dissertação de candidatura ao grau de Mestre em Biologia Molecular Humana apresentada à Faculdade de Ciências da Universidade de Lisboa.

Projecto de investigação desenvolvido no grupo de investigação Neuron Glia Biology in Health and Disease do Centro de Patogénese Molecular da Faculdade de Farmácia da Universidade de Lisboa, integrado no iMed - Research Institute for Medicines and Pharmaceutical Sciences, sob orientação de Dora Brites, PhD com co-orientação de Adelaide Fernandes, PhD, e orientação interna na Faculdade de Ciências de Rui Malhó, PhD.

NOTA DE ABERTURA

Esta dissertação destina-se à obtenção do grau de Mestre em Biologia Molecular Humana, concedido pela Faculdade de Ciências da Universidade de Lisbboa.

O trabalho aqui discutido foi já, em parte, apresentado publicamente nos seguintes congressos:

Adelaide Fernandes, Eduarda Coutinho, Lorene M. Lanier, Dora Brites. *Bilirubin reduces axonal arborization through alterations of cytoskeletal dynamics.* 11th Meeting of Portuguese Society for Neurosciences. Braga, June 4-6 2009. [Poster]

Adelaide Fernandes, Eduarda Coutinho, Lorene M. Lanier, Dora Brites. *Bilirubin-induced changes at neuronal cytoskeletal dynamics: novel cues for reduced axonal arborization.* 9th European Meeting on Glial Cells in Health and Disease. Paris, September 8-12 2009. [Poster]

À minha luz maior, a minha filha **Beatriz,**

que trouxe à minha vida o mais verdadeiro e eterno significado.

A firmeza de propósito é um dos mais necessários elementos do carácter e um dos melhores instrumentos do sucesso. Sem ele, o génio desperdiça os seus esforços num labirinto de inconsistências.

Philip Chesterfield

AGRADECIMENTOS

O sucesso consiste em avançar de fracasso em fracasso sem perder o entusiasmo.

Winston Churchill.

O sucesso não é um acto de solidão; foram muitos aqueles que de alguma forma contribuiram para que eu não perdesse o entusiamo e tivesse a preserverança para concluir esta importante etapa da minha vida. Assim, quero expressar os meus sinceros agradecimentos.

As minhas primeiras palavras vão, naturalmente, para a **Prof. Doutora Dora Brites**, minha orientadora. Agradeço sobretudo por me ter dado a oportunidade de realizar o meu projecto de mestrado no grupo *Neuron Glia Biology in Health and Disease,* agradeço a forma como me acolheu e como se mostrou sempre disponível quando necessitei de conversar consigo. O nivel de exigência e rigor com que pautou a orientação científica deste trabalho, o seu espírito crítico e elevada experiência e conhecimentos científicos foram fundamentais para o sucesso deste projecto e concerteza fizeram de mim uma melhor profissional. A sua dedicação à ciência, a sua capacidade de criação científica e a sua inteligência são inspiradoras. Foi um privilégio trabalhar consigo!

À **Doutora Adelaide Fernandes**, co-orientadora deste trabalho, agradeço a dedicação, o empenho, a constante disponibilidade e o incansável apoio na orientação deste projecto. Saliento as suas excepcionais competências intelectuais, profissionais e científicas, sendo sem dúvida um exemplo de rigor e profissionalismo, que merece a minha admiração. As suas preciosas sugestões, quer na execução técnica quer na redacção desta Tese, foram fundamentais para a qualidade e sucesso deste trabalho. Adelaide, agradeço-te por me teres ajudado a crescer cientificamente e por me fazeres sentir que posso sempre fazer melhor. A tua serenidade transmitiu-me a confiança para acreditar que no fim tudo correria bem. Não posso deixar de te agradecer a simpatia, a amizade e o carinho com que me recebeste no laboratório e com que me presenteaste ao longo deste ano. Aprendi muito contigo. Obrigada!

Ao **Doutor Rui Malhó**, meu orientador na Faculdade de Ciências, agradeço por se ter demonstrado sempre disponível para colaborar neste projecto. Sempre que solicitado, correspondeu o mais prontamente possível. Agradeço a forma concisa e objectiva com que esclareceu sempre as minhas dúvidas e por ter contribuído para o sucesso deste trabalho.

Aos coordenadores do Mestrado em Biologia Molecular Humana, **Doutora Filomena Caeiro** e **Doutor Manuel Carmo Gomes** agradeço o empenho em organizar o mestrado da melhor forma possível, bem como a disponibildade e preocupação em esclarecer as minhas dúvidas da forma mais eficiente.

À **Prof. Doutora Alexandra Brito** e ao **Prof. Doutor Rui Silva** agradeço a forma como me acolheram no grupo. Agradeço os ensinamentos científicos que sempre fluíram tão naturalmente. Destaco a inteligência, o rigor, a preserverança e a paixão pela ciência com que desenvolvem os seus projectos científicos. Agradeço o constante interesse pela evolução do meu trabalho e a disponibilidade sempre demonstrada para colaborar.

À **Doutora Sofia Falcão** agradeço a *boa vizinhança* (☺). Sofia, a tua leveza de espírito é contagiante. Obrigada pela simpatia com que me recebeste no grupo, por desde sempre te mostrares disponível para me ajudar no que fosse preciso e pelos esclarecimentos técnicos e científicos que me prestaste. Tens um sorriso de menina que nos contagia e nos envolve na paixão com que tu vives a tua vida e a tua profissão. Obrigada pela tua amizade, pelas partilhas de *mamãs de primeira viagem* e pela tua constante preocupação com o sucesso do meu trabalho.

Às *nossas* meninas, estudantes de Doutoramento, agradeço a orientação inicial na rotina do laboratório, a simpatia, o bom ambiente de trabalho, o bom-humor, a partilha tão natural dos nossos dias e das nossas vivências. **Ritinha,** a menina *rebelde*, és deliciosamente frontal, dinâmica e competente. **Sandra**, és a verdadeira força da natureza, surpreendentemente resistente, eloquente, organizada. **Andreia,** tão feminina e elegante...mas, surpresa das surpresas...ela joga hóquei! Rigorosa, exigente, aplicada... com olho clínico para formatações (☺). **Inês**, um verdadeiro *mini-break,* tens uma alegria contagiante e desde cedo me mostraste as tuas preciosas qualidades como pessoa e profissional. Mas algo vocês têm em comum, um verdadeiro sentido de amizade. A todas um enorme obrigado pelo vosso apoio e por estarem sempre disponíveis para ajudar no trabalho experimental, aprendi muito convosco! Será com enorme orgulho que daqui a uns tempos assistirei à conquista dos vossos Doutoramentos!

Às minhas companheiras de viagem, as *meninas do mestrado*, **Catarina** e **Filipa**, agradeço a amizade, o espirito de entre-ajuda, a partilha de cada momento, de cada alegria, de cada frustração, mas sobretudo a felicidade de chegar ao fim!!! Parabéns!

À **Ema**...a *minha* pequena Ema, que também pertence ao grupo das *meninas do mestrado*, mas cuja amizade passou os limites do companheirismo. Ao longo deste ano foste o *meu braço esquerdo* (☺), e, embora falando muitas vezes sozinha, foste fazendo com que eu te ouvisse cada vez com mais atenção. Cativaste o meu coração e despertaste em mim um certo sentimento de protecção! Construímos uma verdadeira amizade. Obrigada por tudo aquilo que não cabe em palavras, mas sobretudo por veres para além do evidente, porque às vezes o evidente mente, como sabes! Não esquecerei nunca os momentos de partilha, a boa disposição e as longas horas que passámos juntas a escrever as nossas teses! És uma menina excepcional, convicta dos teus objectivos e com uma enorme capacidade de dedicação! Parabéns por esta MERECIDA conquista. Espero, daqui a uns anos, assistir ao teu

Doutoramento. Desejo que a vida te traga muitas alegrias e te faça manter essa feliz espontaneidade.

À **Cibelle**, nova estudante de mestrado, dou as boas-vindas e desejo muita sorte e empenho para enfrentar os desafios que se avizinham. À **Catarina Osório** e ao **Daniel** que, durante o tempo em que pertenceram ao grupo, me proporcionaram excepcionais momentos de boa disposição e companheirismo. Obrigada pelo vosso apoio, simpatia e amizade. Estarei sempre presente para vos ver alcançar os vossos maiores sonhos. **Daniel**, mostraste que nunca é tarde para seguir um sonho. **Catarina**, obrigada pelas leituras atentas que foste fazendo do meu projecto e sobretudo pelas gargalhadas mais profundas que demos juntas!!!

Aos colegas com quem partilhei a sala *das catacumbas* (☺), **Susana, Rui, Rita, Joana, Filipa, Pedro, Márcia, Ricardo, Benedita, Maria, Inês, Andreia, Mónica, Kátia e Ana**, obrigada por serem bons colegas, por serem bem dispostos, por me terem recebido com tanta simpatia e também por ficarem felizes com as nossas conquistas. Vocês fazem com que o ambiente da nossa sala seja tão bom, tão bom, que por vezes é difícil trabalhar!!! ☺

À minha amiga e colega de aventura desde o início, a **Madalena**, agradeço a amizade, a disponibilidade e apoio constantes e a companhia nos cafés da manhã. Admiro a tua inteligência, o teu empenho e a tua capacidade de trabalho, tenho a certeza que vais chegar muito longe! Obrigada por teres feito parte da minha vida nestes dois últimos anos!

Aos restantes colegas do Centro de Patogénese Molecular agradeço a simpatia, o espirito de entre-ajuda e a alegria com que me dizem bom dia todas as manhãs, fazendo com que o CPM seja um local muito agradável para trabalhar.

Àqueles que há muitos anos me acompanham, os meus maiores amigos. A minha *mana* **Liliana e Henrique**, **Paulinha**, **Susana**, **Clara e Edgar**, **Adriana e João**, **Catarina**, **Carla e Duarte**, **Dora e Luís**, **Julieta**, **Carmito e Dânia.** Vocês preenchem a minha vida de forma muito especial. Têm-me visto crescer como pessoa e têm partilhado da minha construção de vida. É convosco que desabafo, que rio, que choro, que partilho as minhas inseguranças. É no vosso sorriso que encontro a força para continuar e no vosso abraço sinto a verdadeira força da amizade!!! Somos a prova de que os sentimentos não têm fronteiras e são intemporais! Passam-se os anos e sempre que nos encontramos sinto que nada mudou, e nessas alturas tenho vontade que o tempo não passe! Eu costumo dizer que a amizade não se agradece, retribui-se. Mas na verdade eu sinto-me grata por estar rodeada de bons amigos como vocês, e tenho de vos agradecer toda a força, apoio e envolvimento nos meus projectos de vida. Obrigada por compreenderem as minhas ausências nesta fase de trabalho árduo e por esperarem por mim!!!! Adoro-vos de todo o coração, obrigada por fazerem da minha vida uma existência muito mais alegre!!

À minha **família,** o *clã* **Coutinho,** que, embora longe, me tem apoiado da forma que lhes é possível. Obrigada pela preocupação e por serem felizes ao me verem triunfar!!!

Ao meu mano **João**, por ser o mano mais carinhoso que existe! Eu sei que, apesar de estarmos muito tempo longe um do outro, os nossos corações vivem juntos! Obrigada por me incentivares sempre a progredir, por demonstrares que tens orgulho em mim e por ficares feliz com as minhas conquistas! Adoro-te!

Aos meus pais, **mamã Conceição** e **papá Carlos**...não sei como se agradece aos pais! Como é que se agradece às pessoas que nos deram a VIDA?! Certamente não existem palavras suficientes no mundo para expressar a gratidão que sinto pela minha existência, pela forma como me educaram e me ensinaram a valorizar as coisas certas! Obrigada por estarem SEMPRE presentes, por me apoiarem, por acreditarem em mim, pelos sacrifícios que fizeram por mim ao longo da vida. Por terem sido sempre um exemplo de honestidade, integridade e empenho. Obrigada por me ensinarem a lutar pelos meus sonhos e por terem construído a pessoa que sou hoje. Com todo o meu amor, obrigada!!!

Ao meu marido, **João** que literalmente patrocinou esta minha aventura! Sem ti não teria conseguido! Às vezes o amor tem formas estranhas de ser. O amor existiu quando apoiaste a minha decisão de fazer o mestrado, mesmo sabendo que isso iria implicar um grande esforço de ambos. O amor existiu em todas as vezes que me substituiste nas tarefas domésticas e em todas as vezes que foste pai e mãe para a Beatriz. O amor existiu nas vezes em que abdicaste da minha companhia por eu ter de trabalhar. O amor existiu nas vezes em que ouviste os meus desabafos e as minhas inseguranças. O amor existe sempre que me incentivas e acreditas no meu valor. O amor existe sempre que me abraças e me dizes que vai correr tudo bem! Foram dois anos de grande esforço...mas *o que não nos mata torna-nos mais fortes*!!! Obrigada pelo teu apoio incondicional, pela tua compreensão, pela tua amizade...pelo teu amor!

Beatriz, é o nome do meu maior orgulho! A extensão do meu ser, a minha filha, que me ensinou o que é o amor mais incondional e mais indescritível. Obrigada por me dares o privilégio de o sentir! Desculpa todas as vezes em que me disseste *mamã, não estudes mais, binca com a Bia* e a mamã não pôde ir brincar contigo! Certamente foste quem mais sofreu com as minhas ausências durante estes anos, e por isso esta Tese é para ti! Poder-se-ia pensar que por ter a minha filha tudo seria mais difícil, mas não; tudo foi mais fácil...porque tive sempre um bom motivo para sorrir!!! Obrigada pelo teu carinho, pelos abraços e beijinhos mais fofinhos e por me fazeres sorrir incondicionalmente! A mamã tem um cantinho no coração que é só teu, onde mora o amor maior do Mundo!!! E agora, princesa linda... a mamã já pode ir brincar contigo! ☺

TABLE OF CONTENTS

TABLE OF FIGURES

ABBREVIATIONS

APS	ammonium persulfate
ATP	adenosine triphosphate
BAX	bcl-2-associated-x-protein
BBB	blood brain barrier
BHE	barreira hemato-encefálica
BNC	bilirrubina não conjugada
BSA	bovine serum albumin
Ca^{2+}	Calcium
CaMKII	ca2+/calmodulin-dependent protein kinase ii
CNS	central nervous system
CO	carbon monoxide
CO2	carbon dioxide
ºC	degree Celsius
DIV	days *in vitro*
E16	embryonic day 16
EB	end-binding protein
ECL	enhanced chemiluminescence
EDTA	ethylendiaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
FBS	fetal bovine serum
FCS	fetal calf serum
Fig.	figure
g	gram
GFP	green fluorescent protein
GTP	guanosine triphosphate
HBSS	hanks' balanced salt solution
HCl	chloride acid
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HSA	human serum albumin
IgG	immunoglobulin G
IL	interleukin
KIF	kinesin superfamilly
KOH	potassium hydroxide
l	liter
LTP	long term potentiation
M	molar
MAP	microtubule associated protein

MEM	minimun essential medium
mg	miligrams
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
ml	milliliters
mM	milimolar
mm	millimeter
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
Na_2HPO_4	sodium phosphate
NaCl	sodium chloride
nM	nanomolar
p	p value
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffer saline
pH	negative logarithm for hydrogen
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PMSF	phenylmethanesulphonylfluoride
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TBS	tris-buffered saline
TBS-T	TBS-tween
TEMED	tetramethylethylenediamine
TGS	tris-glycine-sds
TIPs	tracking proteins
TNF-α	tumor necrosis factor alpha
UCB	unconjugated bilirubin
UDP	uridine diphosphate
v/v	volume per volume
w/v	weight per volume
µg	microgram
µm	micrometer/micron
µM	micromolar
µmol	micromole
µl	microliter
%	percentage

ABSTRACT

Hyperbilirubinemia is frequently observed in the neonatal period due to the transient accumulation of unconjugated bilirubin (UCB) in serum, which in certain circumstances it may trigger neurological deficits. We have recently shown that exposure of immature hippocampal neurons to UCB, in conditions mimicking moderate to severe neonatal jaundice, impairs neuronal development, with reduced neurogenesis, neurite arborization, and synaptogenesis. This project aimed to appraise the molecular mechanisms by which UCB may induce mild or severe alterations in the establishment of the normal neuronal architecture and inter-neurons communication that may ultimately elicit the appearance of neurological disabilities. As a first step to address this question we evaluated the effect of UCB at the neuronal cytoskeletal dynamics focusing on: (i) axonal elongation, (ii) microtubule polymerization and stabilization, and (iii) axonal transport.

We first observed that UCB exposure lead to a higher percentage of neurons with decreased axonal length. Since microtubules polymerization and stability have crucial roles in axogenesis, we further investigated the polymerization rate of microtubules and found that it was also impaired after UCB exposure. In addition, hippocampal neurons treated with UCB revealed a more stable microtubules array, which may be related to a decreased axonal outgrowth. As our former results demonstrated that UCB increases microtubules stability, we decided to assess the UCB effects on microtubule associated proteins, MAP2 and Tau1. Curiously, we observed that UCB increases MAP2 axonal entry while it also enhances MAP2 and Tau1 expression along the axon, as well as their binding to microtubules. It is known that microtubule-bound Tau1 is able to interfere with the progress of motor proteins such as kinesin and dynein along the axon. Interestingly, in UCB-treated neurons we found a decreased expression of kinesin and a marked elevated expression of dynein, which may compromise the normal axonal transport. One of the cargoes transported via motor proteins are mitochondria. In this regard we demonstrated that UCB exposure induces mitochondria to aggregate and accumulate on the distal portion of the axon, suggesting a deficient axonal transport.

These findings are new clues on the mechanisms of neurologic impairment induced by bilirubin in moderate to severe neonatal jaundice, showing cytoskeleton alterations similar to those often observed in neurological and neurodegenerative diseases.

Keywords: Neonatal jaundice; hippocampal neurons; neuronal architecture; cytoskeleton; microtubule dynamics; axonal transport.

RESUMO

A hiperbilirrubinémia, mais vulgarmente denominada de icterícia, é uma condição clínica frequentemente observada nos primeiros dias de vida de um recém-nascido provocada por uma acumulação transitória de bilirrubina não conjugada (BNC) na circulação sanguínea, devido a um reduzido tempo de vida dos eritrócitos e a uma ineficaz excreção hepática. A bilirrubina é um composto que se forma no sistema retículo-endotelial como produto da degradação da hemoglobina, é transportada na circulação ligada à albumina, sendo posteriormente absorvida pelo fígado onde é conjugada com o ácido glucurónico antes de ser excretada. Os recém-nascidos apresentam uma certa imaturidade da funcionalidade hepática pelo que alguns mecanismos de depuração da bilirrubina (conjugação e secreção) ficam comprometidos originando um aumento da sua concentração a nível sistémico.

Em condições normais, a bilirrubina em circulação encontra-se maioritariamente ligada à albumina e apenas uma fracção extremamente pequena atravessa a barreira hemato-encefálica (BHE). No entanto, em situações de hiperbilirrubinémia moderada ou severa a fracção não ligada à albumina aumenta e maior quantidade acede ao cérebro. Pelas suas características de insolubilidade a BNC acaba por se depositar no sistema nervoso central, o que pode conduzir a sequelas neurológicas reversíveis ou irreversíveis e até mesmo à morte, quadros clínicos designados por encefalopatia bilirrubínica e k*ernicterus*, respectivamente. Estas situações são exacerbadas em bebés prematuros que têm uma BHE mais permeável e uma actividade enzimática do fígado ainda mais reduzida, além de que apresentam menor tolerância à neurotoxicidade da BNC e ainda uma maior incidência de processos patofisiológicos que podem contribuir para o agravamento do dano cerebral.

Os mecanismos de neurotoxicidade induzidos pela exposição à BNC não são de todo conhecidos; no entanto, o nosso grupo tem-se dedicado a dissecar muitos dos processos celulares e moleculares que possam ser alvo da acção neurotóxica da BNC nas células do sistema nervoso central. Os nossos estudos já demonstraram que a neurotoxicidade induzida pela acção da BNC é dependente do tipo celular, do estado de diferenciação das células, bem como do nível e do tempo de exposição. Recentemente demonstrámos que uma exposição precoce à BNC compromete o neurodesenvolvimento, reduzindo a neurogénese, a ramificação das neurites e a sinaptogénese. Estes dados sugerem que a BNC actua em processos como a diferenciação neuronal, comprometendo a funcionalidade e conectividade dos neurónios.

O citosqueleto neuronal tem um papel crucial na morfogénese e polarização dos neurónios, que são células morfologicamente e funcionalmente peculiares. É uma estrutura

constituída sobretudo por microfilamentos e microtúbulos, elementos dinâmicos que interagem entre si e com outras estruturas celulares. Os componentes do citosqueleto incluem proteínas reguladoras e associadas aos microtúbulos (*microtubule associated protein* - MAP) que contribuem para a sua estabilização, proteínas ligas à actina e proteínas motoras, como a quinesina e a dineína.

A formação do axónio marca o início do processo de polarização neuronal e requer uma maior estabilização dos microtúbulos, os quais apresentam nesta fase uma maior resistência à despolarização. No entanto, durante o crescimento axonal deve existir um certo nível de instabilidade dos microtúbulos, um processo designado por instabilidade dinâmica, para que segundo ciclos de polarização e de despolarização formem novas unidades necessárias para a construção da matriz do citosqueleto axonal.

Duas das proteínas associadas aos microtúbulos são a MAP2 e a Tau1, que têm localizações constitutivas distintas, enquanto a MAP2 se encontra sobretudo no corpo celular e dendrites, a Tau1 situa-se mais no axónio, para além de que ambas têm a capacidade de interferir com a ligação das proteínas motoras aos microtúbulos. Outra classe importante de MAPs são as +TIPs (*microtubule plus-end tracking protein)* que são mais especializadas e reconhecem as extremidades dos microtúbulos polimerizadas *de novo*, como a EB3 (*end-binding protein-3*). O estado de fosforilação destas proteínas também altera a sua capacidade de ligação aos microtúbulos. Estas proteínas associadas aos microtúbulos são certamente fortes candidatas a reguladores da organização e funcionamento do citosqueleto neuronal.

O presente projecto pretende dissecar alguns dos mecanismos moleculares pelos quais a hipebilirrubinémia pode induzir alterações moderadas ou severas no estabelecimento da arquitectura neuronal, bem como na comunicação inter-neuronal, que em última análise podem conduzir ao aparecimento de disfunções neurológicas. Para tal fomos avaliar o efeito da BNC na dinâmica do citosqueleto neuronal, focando: (i) o crescimento axonal, (ii) a polimerização e estabilização dos microtúbulos, e (iii) o transporte axonal.

Utilizámos culturas primárias de neurónios de hipocampo como modelo de estudo. Estas células foram isoladas a partir de embriões de murganho com 16 dias de gestação (E16) e cultivadas sobre poli-D-lisina. Após 1 dia *in vitro* (DIV), as células foram incubadas durante 24 h com meio de cultura incluindo albumina sérica humana (100 μM), aqui designado por veículo, ou com BNC (50 μM e 100 μM). Estas concentrações de BNC pretendem mimetizar situações de hiperbilirrubinémia moderada e severa, respectivamente. As células foram analisadas aos 3 DIV. Para identificar as proteínas que nos propusémos a estudar (MAP2, Tau1, EB3, quinesina e dineína) utilizámos uma técnica de imunofluorescência que nos permitiu avaliar a expressão e localização da proteína ao longo

do axónio. Os axónios foram traçados manualmente e a intensidade de fluorescência relativa à expressão de cada proteína foi medida recorrendo ao software ImageJ v1.40.

Inicialmente avaliámos a acção da BNC no crescimento axonal e observámos que a percentagem de neurónios com comprimento inferior a 100 µm aumentou (30%) relativamente às células tratadas apenas com o veículo (20%), resultando num tamanho médio axonal igualmente reduzido (~10%, $p<0.05$ e ~15%, $p<0.01$, para 50 e 100 µM de BNC, respectivamente). Estes dados apontam para um efeito nocivo da BNC na formação do axónio. Posto isto, resolvemos analisar qual o efeito da BNC ao nível da polimerização dos microtúbulos. A análise da fluorescência da EB3 ao longo do axónio demonstrou uma redução significativa na presença de EB3, sobretudo na zona apical do axónio, em células tratadas com BNC (~30% e ~25%, $p<0.05$ para 50 e 100 µM de BNC, respectivamente), o que pressupõe uma diminuída polimerização dos microtúbulos.

Uma vez que o crescimento axonal necessita que os microtúbulos apresentem um certo grau de despolimerização, e tendo observado que a exposição à BNC induz uma redução quer na polimerização dos microtúbulos, quer no comprimento axonal, tornou-se pertinente investigar se a BNC estaria a actuar ao nível da estabilização destas estruturas. As células foram postas em contacto com vimblastina, um potente destabilizador dos microtúbulos, durante 2 h imediatamente antes de serem fixadas para a análise de imunofluorescência. Como seria de esperar, as células expostas à vimblastina apresentaram um decréscimo no comprimento axonal de ~20% ($p<0.001$). Curiosamente, os neurónios tratados com BNC também evidenciaram redução no tamanho do axónio de ~10% ($p<0.05$) e de ~15% ($p<0.01$) para 50 e 100 µM de BNC, respectivamente. No entanto, quando incubadas em simultâneo com BNC e vimblastina, as células não apresentaram maior redução do comprimento axonal, demonstrando que a BNC estará a provocar uma maior estabilização dos microtúbulos de uma forma não reversível pelo efeito da vimblastina.

Adicionalmente, fomos avaliar a expressão da MAP2 e da Tau1, bem como a sua capacidade de ligação aos microtúbulos. A exposição dos neurónios à BNC induziu uma maior entrada da MAP2 no compartimento axonal de uma forma dependente da concentração (~14, $p<0.01$, para 50 µM de BCN e ~ 16%, $p<0.001$, para 100µM de BNC). A MAP2 ao longo do axónio apresenta-se também aumentada, sobretudo na região apical (~30%, $p<0.05$ e ~70%, $p<0.001$, para 50 e 100 µM de BNC, respectivamente), assim como a sua ligação aos microtúbulos nas zonas intermédias (~40%, $p<0.001$ para 100 µM de BNC) ou a nível mais distal do axónio (~25%, $p<0.01$ para 50 µM de BNC).

A expressão de Tau1 aparece também alterada pela acção da BNC, observando-se um aumento significativo com 50 µM de BNC (>60%, $p<0.001$), mas um decréscimo, igualmente significativo, com 100 µM de BNC ((~20%, $p<0.001$). Curiosamente, quando avaliámos a ligação de Tau1 aos microtúbulos encontrámos um aumento induzido por

ambas as concentrações de BNC (~40%, p<0.01 e >80%, p<0.001 para 50 e 100 μM de BNC, respectivamente). Estes dados sugerem que a BNC parece interferir na integridade estrutural e funcional do citosqueleto.

Estas alterações estruturais podem, sem dúvida, comprometer o transporte axonal. De facto, observámos igualmente que os neurónios expostos à acção da BNC apresentavam alteração do padrão de expressão das proteínas motoras, quinesina e dineína. A BNC provocou uma diminuição da expressão da quinesina de forma proporcional à sua concentração, atingindo valores significativos com 100 μM de BNC (~10%, p<0.05). Por outro lado, as duas situações de hiperbilirrubinémia por nós mimetizadas produzem efeitos contrários na expressão da dineína. Enquanto que a expressão de dineína foi significativamente diminuída por 50 μM de BNC (~10%, p<0.05), perante 100 μM de BNC a sua expressão foi bastante aumentada (~40%, p<0.001). Estes dados sugerem que uma exposição à BNC poderá comprometer o transporte axonal.

As mitocôndrias são um dos exemplos de organelos celulares que é transportado por estas porteínas, as quais estão envolvidas em processos metabólicos fundamentais para a sobrevivência e funcionalidade celular. Após todas as observações acima descritas, quisémos perceber até que ponto todas as alterações induzidas pela BNC alteravam a distribuição axonal das mitocôndrias. De facto, observámos que a BNC induz a formação de aglomerados mitocondriais, localizados numa região intermédia-distal do axónio quando numa concentração de apenas 50 μM BNC (~25%, p<0.05) ou na porção distal do axónio quando numa concentração de 100 μM (>55%, p<0.05). Uma vez mais, estes dados apontam para um deficiente transporte axonal após exposição à BNC.

Em conjunto, os nossos resultados esclarecem algumas das alterações induzidas na estrutura do citoesqueleto de neurónios imaturos quando expostos a situações de hiperbilirrubinémia moderada a severa, contribuindo para um melhor conhecimento das repercussões da encefalopatia por bilirrubina no período neonatal relativamente ao desenvolvimento de patologias neurológicas e/ou neurodegenerativas que podem emergir numa fase mais avançada da vida.

Palavras-chave: Icterícia neonatal; neurónios de hipocampo; arquitectura neuronal; citosqueleto; dinâmica dos microtúbulos; transporte axonal.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. HIPPOCAMPUS LOCALIZATION AND FUNCTION

The limbic system consists of medial temporal lobe structures including hippocampal formation, amygdala, the parahippocampal gyrus and the cingulate gyrus (Fig.1). These cortical areas were named *limbic* because they form a border (*limbus*) around the inner structures of the diencephalon and midbrain. The hippocampal memory system includes the hippocampus, orbital and medial prefrontal cortex, ventral parts of the basal ganglia, the mediodorsal nucleus of the thalamus and also the amygdala [1]. The anatomy and circuit of the hippocampus are extremely conserved across mammalian species [3,4].

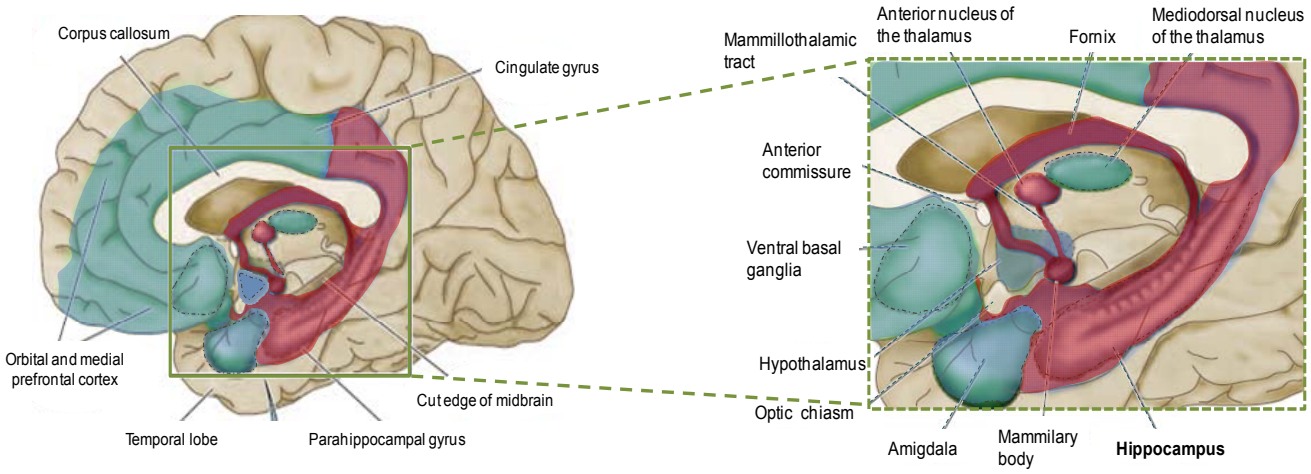


Fig.1. Actual conception of the limbic system. The most notorious components are the cingulate gyrus, the amygdala with important role in experience and expression of emotion, and the hippocampus which function is mainly related with learning and memory processes. Adapted from Purves *et al.*, 2004 [1].

The hippocampus and parahippocampal gyrus play an important role on learning and memory process. Rodents and primates depend on these medial temporal structures to encode and consolidate memories of events and objects in time and space, while humans also use these same brain regions for the initial encoding and consolidation of short-term declarative memories [1]. Lesions of hippocampus and in parahippocampal region in humans induce profound anterograde amnesia that is characterized by an inability to retain and consciously recollect memories of facts and events [3]. In young monkeys, hippocampal injury result in deficient social interactions early in life, which become more severe during adulthood [4]. Furthermore, there are evidences of hippocampal pathology in schizophrenia with morphological, molecular and functional aspects (for review see Harrison, 2004 [5]).

1.2. HIPPOCAMPAL NEURONS

Neurons are the basic information processing units of the central nervous system (CNS). Neurons are very specialized and polarized cells that can be categorized according to their size, shape, location, neurochemical characteristics and connectivity, which is determinant to its function in the brain. Morphologically it is possible to identify three main regions in a typical neuron: (1) the cell body with the nucleus and the major cytoplasmic organelles, (2) a variable number of dendrites that receive electrical signals and (3) a single axon that extends far from the cell body transmitting electrical signals. In addition to signal processing and propagation, the axonal and dendritic surfaces also play *house-keeping* roles such as receiving and releasing different molecules from and into their environment. In addition, the elaborated branching morphology of axons and dendrites enable neurons to form complex signaling networks [3,6-8]. One of the earliest and best studied neuronal culture systems is the growth of hippocampal neurons from rat embryonic brain. This system has shown that neurons develop their characteristic morphology through a stereotypic sequence of events with distinct intermediate steps (Fig.2).

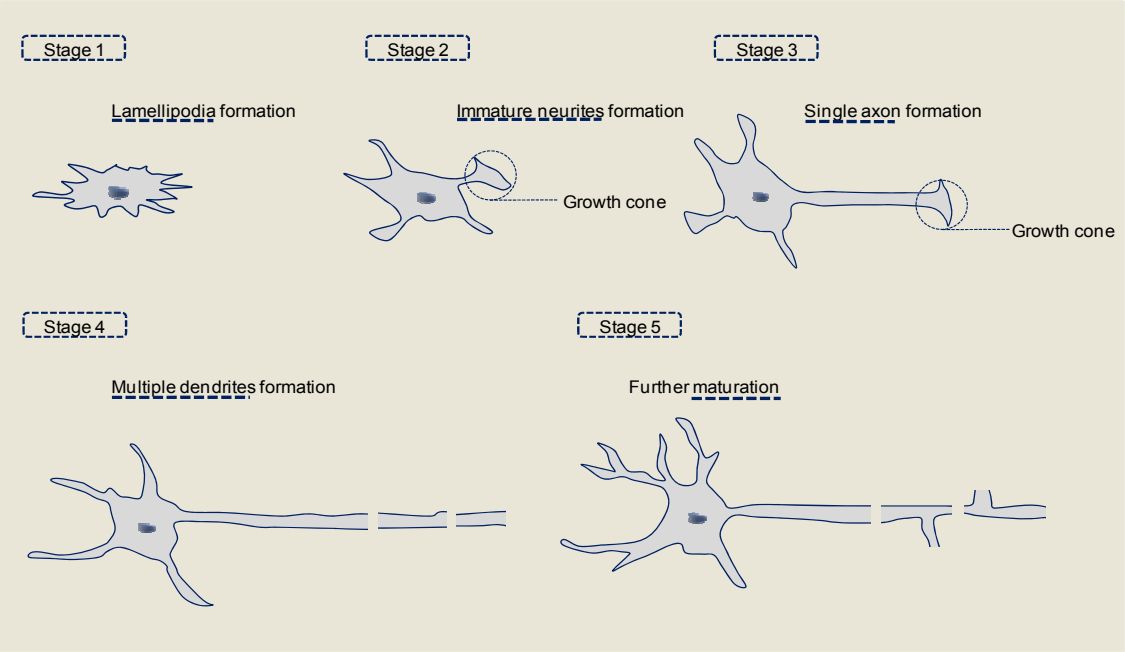


Fig.2. Schematic representation of hippocampal neurons developmental stages. Hippocampal neurons *in vivo* and *in vitro* develop in a characteristic sequence of stages that start with the formation of a *lamellipodia* structure and mature until dendrites and single axon are formed. Adapted from Yoshimura *et al*., 2006 [2].

After initially extending *lamellipodia* (stage 1), the cells elaborate several short, *minor* processes that are roughly equal in length and cannot be identified as axonal or dendritic (stage 2). After a period of 6 to 24 h the cell undergoes its initial polarization. At this time, one of the minor processes begins to grow rapidly, becoming many times longer than the others within just a few hours (stage 3) and acquires molecular, structural and functional

characteristics to become the neuronal axon. A few days later, the remaining minor processes also begin to grow but at a significantly slower rate than the axon. These processes gradually acquire dendritic characteristics and become the neuronal dendrites (stage 4). Finally, the axons and dendrites of neighboring neurons develop extensive synaptic connections and establish a neuronal network (in stage 5) [2].

1.3. CYTOSKELETAL FUNCTION AND DYNAMICS ON NEURONAL ARCHITECTURE

The cytoskeleton of eukaryotic cells consists of an aggregate structure formed by three classes of cytoplasmic structural proteins: microtubules, microfilaments and intermediate filaments. All of these structural elements are dynamic, interacting with each other and with other cellular structures. The cytoskeleton is biochemically specialized for each particular cell type, function and developmental stage[3]. Cytoskeletal components also include cytoskeleton-regulating proteins such as microtubule-associated proteins (MAPs), actin-binding proteins and ‘‘motor’’ proteins. In neurons, all these components play important roles in the establishment and maintenance of neuronal polarity, cell morphology and intracellular transport [9,10]. In fact, distinct microtubule patterns of axons and dendrites result in the routing of different cytoplasmic organelles into each type of neurite thus contributing directly to the establishment of neuronal polarity [6,8,11].

1.3.1. Microfilaments

Microfilaments form twisted strands of actin subunits and in neuronal cells are short, ranging from 200 to 250 nm in length. They have important roles in cell contractility and in local trafficking of both cytoskeletal and membrane components[3]. Actin is a major component of the cellular cytoskeleton and one of the most abundant cellular proteins. It is precisely regulated during many essential cellular functions as migration, adhesion or division. Actin also forms the core of many cellular structures as *filopodia* or *lamellipodia*. In parallel to microtubules, actin dynamics also have a key regulatory role during axon formation process, once local actin instability may cause reduced obstruction of microtubule projection and consequently, of neurite outgrowth. Actin dynamics can also create changes in axonal transport and, therefore, actin dynamics and microtubule stabilization might together coordinate neuronal polarization [12,13].

1.3.2. Microtubules

Microtubules are highly dynamic polar filaments composed of 13 protofilaments, each consisting of a linear array of α/β tubulin dimmers. They polymerize in an asymmetric

manner, resulting in a fast-growing (*plus*) end and a slow-growing (*minus*) end. Interestingly, axons contain a parallel array of microtubules oriented with their plus ends distal to the cell body, while dendrites contain an antiparallel array of microtubules in which only half of the microtubules are oriented with their minus ends distal to the cell body [14,15].

The axon formation is one of the initial steps in breaking cellular symmetry and in the establishment of neuronal polarity. Microtubule stabilization has an active function during the specification of axonal fate in early neuronal development, since axon formation correlates with increased microtubule stability, having the axonal microtubules a higher resistance to depolymerization. Indeed, it was recently shown that the presence of a microtubule stabilizer such as taxol near a growing neurite may promote its development into an axon [16].On the other hand, during axonal elongation microtubules suffer cycles of growth and collapse termed *dynamic instability*, in which, at their plus-end, microtubules frequently repeat transitions from *catastrophe* (from polymerization to depolymerization) to *rescue* (from depolymerization to polymerization) [17,18]. In this case, the presence of endogenous microtubule destabilizing factors is crucial for the correct axonal outgrowth since it allows a certain degree of microtubule instability. Nevertheless, their concentration must be finely regulated since it was reported that a decrease of their expression may suppress neurite outgrowth, while their excess may induced neurite retraction by an excessive microtubule disassembly [18].

Thus, the presence of destabilizing factors such as vinblastine, and nocodazole, which suppress the dynamic instability of microtubules ends, may reduce the rate of neurite elongation [16,18-20].

1.3.3. Microtubule associated proteins

The stabilization of microtubules is helped by classical microtubule associated proteins (MAPS) which can bind to microtubule frames to increase the polymerization rate and form cross-bridge structures between microtubules and also between microtubules and other cytoskeletal elements [21]. In the nervous tissue, MAPS are constituted by two heterogeneous categories: Tau proteins and high molecular weight MAPS. The last ones are expressed early during neuronal differentiation and axon development and have been implicated in regulating neurite outgrowth [3]. Microtubule-associated protein 2 (MAP2) is an abundant neuron-specific protein, mainly localized on cell soma and dendrites, that binds to microtubules through a domain near its carboxyl terminus [22,23]. The multiple human MAP2 isoforms are expressed from alternative splicing of a pre-mRNA transcribed from a single gene [22,24]. It is likely that phosphorylation at distinct sites of the molecule differentially affects MAP2 function, modulating its interaction with microtubules and, as a consequence, its capability to stabilize them [25].

Tau also exists in different isoforms, which are generated from a single mRNA by alternative splicing with additional heterogeneity produced by phosphorylation and are expressed differentially through different regions of nervous system and developmental stage. For instance, low molecular weight Tau1 isoform (60 to 75 KDa) is mainly expressed in adult CNS, whereas an isoform of 100 KDa occurs in the peripheral nervous system. Tau binds to microtubules during assembly and disassembly cycles and promotes microtubule assembly and stabilization [3]. In this regard, Tau as also been implicated in the outgrowth of neural processes and in the development of neural polarity. The phosphorylation events that occur on Tau´s structure can change its conformation leading to a decreased microtubule binding by reducing its affinity to the microtubules array, being considered the cause of neuronal dysfunction in neurodegenerative disorders [26,27].

The neuronal MAP2 and Tau are candidate regulators of the organization of the neuronal cytoskeleton [28]. Although in the embryonic brain, fetal Tau (the smallest isoform) and other MAPs are initially homogeneously distributed, Tau and MAP2 are characteristically distributed *in situ*, with Tau localized in the axon and MAP2 somatodendritically compartmentalized, as represented on Figure 3 [29]. Caceres and collaborators (1992) suggested that MAP2 and Tau have distinct functions in neuronal morphogenesis: the initial establishment of neurites depends upon MAP2, whereas further neurite elongation and cell polarization depends upon Tau and microtubule stabilization [30]. Tau expression increases strongly during brain development, concomitantly with neurite outgrowth. Binding of Tau to the distal axon precedes microtubule assembly at this position indicating a function of Tau in organizing microtubules at the distal axonal shaft, and at the onset of axon formation. On the other hand, MAP2 interaction shifts from an actin-associated to a microtubule-associated pattern, suggesting that MAP2 potentially links the actin skeleton to the microtubule network which may be an important event for the initiation of process formation. Hence, it seems that both Tau and MAP2 are required for establishing neuronal polarity [31,32].

A very specialized class of MAPs, the microtubule plus-end tracking proteins (+TIPs), has recently received a lot of attention in this context. +TIPs comprise structurally unrelated proteins including microtubule motors, motor-cargo adaptors and non-motor MAPs. +TIPs either preferentially recognize the structure of the freshly polymerized microtubule end or copolymerize with tubulin subunits providing an interface for the interaction of microtubules with the actin cytoskeleton. In this regard, they play an important role in intracellular transport and positioning of cellular structures [33]. The kinetics of association and release may be regulated by post-translational modifications of the +TIP, for example, phosphorylation. Imaging studies of +TIPs fused to enhanced green fluorescent protein (EGFP) showed to be excellent tools for studying microtubule growth dynamics in living cells [34]. One member of +TIPs proteins is endogenous end-binding protein (EB) 3 , that is expressed preferentially in

the CNS and, as a homologue of EB1, it can be associated with the plus ends microtubule cytoskeleton during assembly (Fig.3) [35]. The excursion of the EGFP-EB3 at the plus end of the microtubule appears as a moving *comet* because of the gradual dissociation of EB3 molecules from the microtubule as it continues to add more subunits and can be used as an excellent marker of microtubule polymerization rate [36].

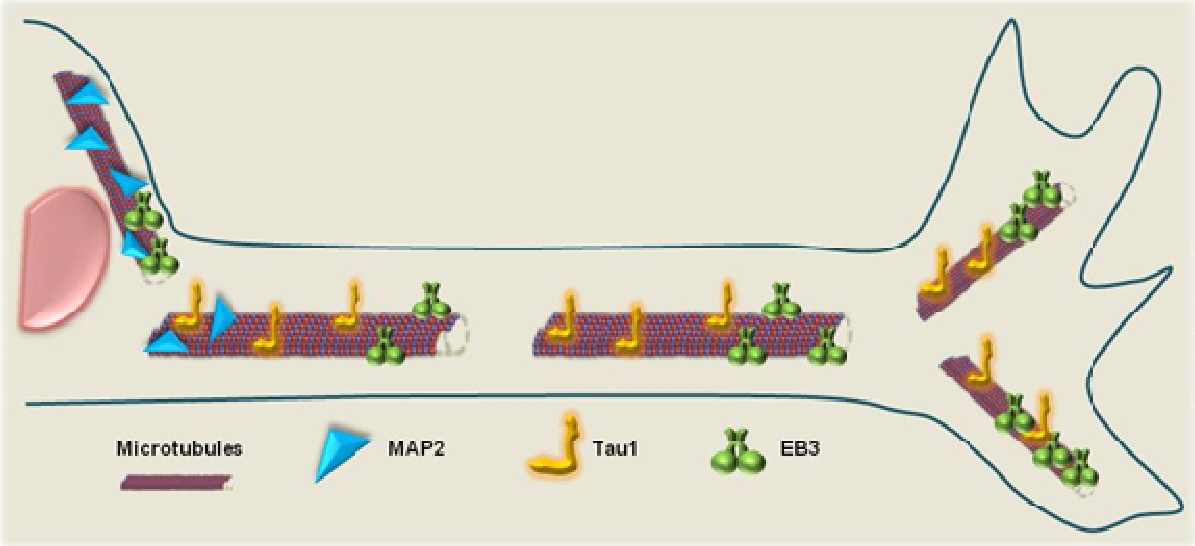


Fig.3. Schematic representation of microtubule associated proteins localization. Microtubule associated protein-2 (MAP2) is localized mainly in cell body and dendrites, while Tau1 is more localized on axonal compartment and endogenous end-binding protein 3 (EB3) binds to plus-ends of newly polymerized microtubules.

1.4. AXONAL TRANSPORT AND MOTOR PROTEINS

Active transport of cytoplasmic material along microtubules is critical for cell organization and function. The basic components of the microtubule-dependent transport system are motor proteins and the polarized microtubule tracks along which they carry cargoes. Anterograde axonal transport moves materials from the cell body out to the synapse and is performed by kinesins, whereas retrograde axonal transport, generated by dyneins, moves materials such as organelles, vesicles, and perhaps neurotrophic signals, from the synapse to the cell body (Fig. 4). The importance of intracellular transport to neurons, suggested by the diversity of transported cargoes and the complexity and specialization of the transport system, implies that the disruption or malfunction of transport could lead to disease [37,38].

Within the cell, the balance between oppositely directed transports determines the steady-state distribution of organelles and biomolecules. In the crowded cell environment, dynein and kinesin compete with non motile MAPs for binding to the microtubule surface. Since dynein travels towards the minus ends and the kinesin superfamily is mostly plus-end directed the polarity and organization of microtubules imparts transport directionality [39].

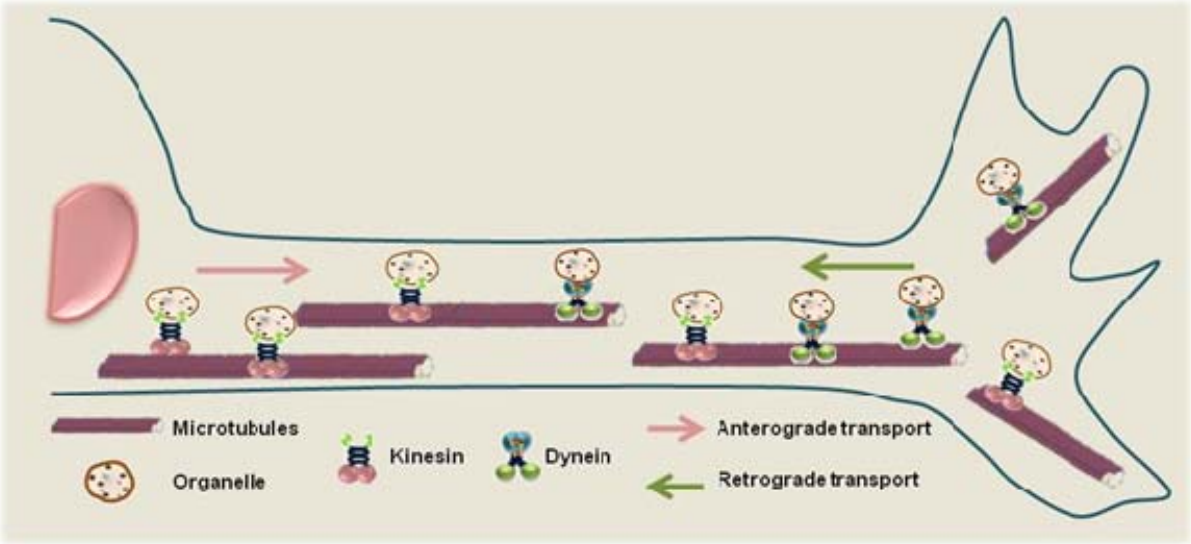


Fig.4. Schematic representation of motor proteins movement in the axon. Kinesin promotes anterograde transport while dynein promotes retrograde transport of organelles along the microtubule tracks.

Interestingly, MAP2/Tau family of proteins can inhibit kinesin and dynein dependent transport along microtubules. Observations in vitro suggest that this inhibition of microtubule motor activity occurs by direct competition of MAP2/Tau proteins with dynein and kinesin for microtubule binding, with kinesin more sensitive to this regulation [40]. This leads to a predominance of retrograde flow and net inhibition of anterograde flow, so that vesicles and organelles tend to accumulate in the cell body resulting in growth cone starvation and decay of cell processes. These organelle clusters first appear at distal endings of axons and may indicate an early stage of neurite degeneration [31,41].

In a healthy neuron, Tau is distributed in a proximal-to-distal gradient. Near the cell body lower Tau concentration allow kinesin to efficiently bind to microtubules and initiate anterograde transport of cargo, whereas higher Tau concentration at the synapse facilitates cargo release. Most attractive is the fact that during a pathologic condition as Alzheimer's disease, Tau accumulates in the cell body and its concentration gradient is disrupted leading to neurodegeneration [42]. In addition, it is described that during tauopathies, including Alzheimer's, Tau dissociates from axonal cytoskeleton causing the microtubule array to gradually disintegrate impairing axonal transport [27].

1.4.1. Mitochondria function and mobility in neurons

Mitochondria are cellular organelles that reside in the cytosol of most eukaryotic cells occupying a substantial portion of cytoplasmic volume. Their main function is the production of energy by oxidizing organic acids and fats with oxygen through oxidative phosphorylation, which may generate oxygen radicals (reactive oxygen species) as a toxic byproduct [43].

In healthy cells mitochondria are really dynamic and plastic organelles experiencing morphological changes by constantly fusion and fission of each other. Mitochondrial fission allows for cellular redistribution of mitochondria in response to local changes in the demand for ATP, whereas mitochondrial fusion enables exchange of mitochondrial DNA and other essential components [44].

To maintain their mobility, mitochondria appear often associated to microtubules network and in neurons they can change between anterograde and retrograde movement, if they must go to the sites of action in axon or to the cell body for degradation and recycling, respectively [7,45]. Although, in mature neurons only one third of axonal mitochondria are mobile, they accumulate in the vicinity of active growth cones and branches in developing neurons, nodes of Ranvier, myelination boundaries and synaptic terminals [46]. This complex pattern of axonal mitochondria mobility suggests that they might be attached to two opposing motor proteins and docking mechanisms. Indeed, it is believed that the specific transport and delivery of mitochondria forward microtubule-based trafficking machinery is guaranteed via indirect linkage with adaptor molecules [47].

Neuronal mitochondria play key roles in the: (a) promotion of microtubule polymerization, by reducing the Ca^{2+} concentration at the presumptive axon which leads to a rapid axonal growth and differentiation; (b) development and synaptic plasticity; (c) arbitration of cell survival and death by necrosis or apoptosis mechanisms; and (d) protein phosphorylation reactions that mediate synaptic signaling and related long-term changes in neuronal structure and function. Hence, it is obvious that mitochondria dysfunction, either due to mutations in mitochondrial genes or mitochondrial impairment, may be consider as a relevant issue in the pathogenesis of prominent neurological/psychiatric disorders, such as dementia or schizophrenia, and neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington's and amyotrophic lateral sclerosis [48].

1.5. NEONATAL HYPERBILIRUBINEMIA: FROM BILIRUBIN METABOLISM TO NEUROTOXICITY

Neonatal hyperbilirubinemia is frequently observed in newborns within the first week following birth due to the transient accumulation of unconjugated bilirubin (UCB). In some cases, especially in infants who are premature and/or have associated hemolytic disorders, UCB levels can increase drastically. In this situation, high free bilirubin (non conjugated and not bound to albumin) levels cross the blood brain barrier (BBB) and precipitate in the CNS. This condition may cause reversible or irreversible alterations, bilirubin encephalopathy or *kernicterus*, respectively, which may contribute to increased morbidity and adverse neurologic *sequelae* [49].

The classical *sequelae* of excessive neonatal hyperbilirubinemia comprise a tetrad consisting of athetoid cerebral palsy, deafness or hearing loss, impairment of upward gaze, and enamel dysplasia of the primary teeth, and correspond to pathologic lesions in the globus pallidus and subthalamic nucleus, auditory and oculomotor brainstem nuclei. Other areas typically damaged by UCB are the cerebellum, particularly the Purkinje's cells, and the hippocampus, which may be affected in a different locus than other disorders, for example hypoxia-ischemia and epilepsy [49,50]. Recently it was described that moderate levels of UCB may cause minor neurologic dysfunction and be correlated with developmental delay, attention-deficit disorder, autism [51]. It have been also connected with the emergence of neuropsychiatric disorders, including schizophrenia [52], as confirmed with an observed greater incidence of Gilbert's syndrome, which indicates an exposure to moderate hyperbilirubinemia, in patients with schizophrenia, when compared to patients with other psychiatric disorders, or with the general healthy population [53-55].

Since the transport of free bilirubin across the BBB is slow, neurotoxic effect of UCB depends directly of the amount of free bilirubin in the brain and the extent of exposure to UCB at different stages of neurodevelopment. In this regard, neurons undergoing differentiation are particularly susceptible to injury by UCB [56] while extremely low birth weight infants, with a peak of total serum bilirubin concentrations during the first 2 weeks of life, show increased death or neurodevelopmental impairment, hearing impairment, and low psychomotor developmental index [57]. These data suggest that prematurity predisposes infants to UCB encephalopathy, considering that preterm newborns have raised susceptibility to UCB brain injury during moderate to severe neonatal jaundice [56,58-63].

Accordingly, the relative imbalance of these processes determines the pattern and degree of neonatal hyperbilirubinemia. All this conveys to a clinical condition designated *physiologic jaundice* [61,64,65]. Commonly, full-term newborns have a peak serum total bilirubin concentration of 5 to 6 mg per deciliter (86 to 103 μmol per liter). Overblown physiologic jaundice occurs at values upon this threshold (7 to 17 mg per deciliter [104 to 291 μmol per liter]) [60,61]. One of the most common treatments used for hyperbilirubinemia is phototherapy, which prevents bilirubin levels from reaching values at which exchange transfusion is necessary. This therapy uses light energy directly in the skin, leading to the isomerization of the UCB molecule when exposed to light, resulting in water soluble molecules that may be excreted in the bile without suffering glucuronidation [65,66].

1.5.1. Bilirubin metabolism

Bilirubin is the final product of heme catabolism, formed in the reticuloendothelial system in which the heme oxygenase cleaves the porphyrin ring of heme, to form the water-soluble biliverdin, a green linear tetrapyrrolic pigment, which is then reduced by biliverdin reductase

to bilirubin, a yellow-orange pigment [67]. The cleavage of the heme yields equimolar parts of biliverdin, iron, and carbon monoxide (CO), while iron is conserved for new heme synthesis, and most of the CO is excreted by the lungs [65,68]. Although 75% to 90% of UCB is derived from the breakdown of hemoglobin from senescent or hemolyzed red blood cells, the other 25% to 10% is produced from de breakdown of other proteins, like myoglobin, cytochromes, catalases and peroxidases [64,65].

Due to its structure, in which internal hydrogen bonding masks the propionic acid side chains, UCB is poorly soluble in aqueous medium (<70 nM), and not readily excreted in the bile or urine. Normally, over 99.9% UCB is transported in the blood tightly bound to albumin, a carrier molecule with a single high affinity binding site for one UCB molecule, and it must be glucuronidated before being excreted in the bile. Subsequent to the uptake by the hepatocyte (which occurs via carrier mediated mechanisms), the pigment is transported by a protein carrier, the glutathione S-transferase. Once within the endoplasmic reticulum, UCB is conjugated with either one or two molecules of glucuronic acid, by the action of the enzyme bilirubin UDP-glucuronosyl transferase [65,67,68].

While during intrauterine life the excretion of UCB is performed through the placenta from the fetus to the mother, at birth, this event is abruptly disrupted leading to an increase in the circulating UCB [61,64]. In addition, during neonatal life, babies have an increased production of UCB due to higher volume of circulating red blood cells per kilogram and a shorter life span of the erythrocytes [65,69]. Since, newborns also present decreased transport and hepatic uptake, decreased conjugation and decreased excretion of bilirubin [65], these situations will lead to a net accumulation of UCB. In preterm newborn, all these processes are even more exacerbated when compared to full-term infant. Indeed, premature babies have reduced liver enzymatic activity [64,70], have a more permeable BBB to UCB, lower tolerance to UCB neurotoxicity [71] and additionally show higher incidence of pathophysiological processes that may account to increased brain damage [72].

Bilirubin bound to albumin probably does not cross the BBB as long as this is intact [66]. However, conditions that alter the BBB permeability, such as infection, acidosis, hyperoxia, sepsis, prematurity, and hyperosmolarity, may increase UCB entry into the brain. Once in the brain, precipitation of free UCB may have toxic effects [60,61].

1.5.2. Mechanisms involved in bilirubin neurotoxicity

Although, the exact cellular mechanism(s) of bilirubin toxicity remain unclear, exposure of neurons to high levels of UCB has been shown to induce apoptosis and/or necrosis associated with mitochondria dysfunction, namely through mitochondrial membrane permeabilization and release of cytochrome *c* [73], Bax translocation to mitochondria, PARP degradation and caspase-3 activation [74] and disruption of the proton gradient required for

oxidative phosphorylation [62]. We have also shown that UCB, at pathophysiologically concentrations, interacts directly with membranes affecting lipid polarity and fluidity, protein order, and redox status [74,75].

In addition, our group also demonstrated that UCB induces an immunologic response in brain cells, enhancing the release of TNF-α, IL-1β and IL-6 by neurons, astrocytes and microglia, this last with a higher magnitude of response [69], through the activation of MAP kinase and NF-κB signal transduction pathways [76]. Moreover, we observed that neurons are more vulnerable than astrocytes to UCB-induced cell death [69] and oxidative injury [77], while astrocytes were reported to be more susceptible to mitochondrial and glutamate uptake dysfunction [78]. Other studies performed in neuronal and astroglial culture models point out that UCB induces greater cell death in more immature cells than in older cultures [58,79,80]. These data suggest that UCB neurotoxicity depends on the cell type and cell maturation state, which is a relevant issue in the increased susceptibility of premature babies [81].

A previous study of our group observed neuronal cytoskeletal disassembly using much higher UCB to human serum albumin ratio [78]. Surprisingly, we also found that early exposure to UCB leads to impairment of neuronal development, with reduced neurogenesis, neurite arborization, and synaptogenesis [59]. In agreement, others argued that UCB may affect learning and memory since even short-term exposure to UCB can inhibit long-term potentiation (LTP) of synaptic transmission [82] and impair neurotransmitter metabolism/release [83] in the hippocampus [84]. These changes may be the basis of neuronal dysfunction and therefore justify the association between neonatal hyperbilirubinemia and the later development of mental illness, such as schizophrenia. This notion is further supported by a recent study using Gunn rats, an animal model of *kernicterus*, in which they demonstrated that these animals present a schizophrenia-like behavioral phenotype [85].

Taking in consideration all the above mentioned effects, it seems that hyperbilirubinemia may act during neurodevelopment altering neuronal differentiation, outgrowth, connectivity, and functionality. Since these events are crucially dependent on accurate cytoskeletal dynamics, it is reasonable to hypothesize that UCB may affect microtubule rearrangements and consequent neuronal function. Curiously, Okumus and collaborators (2008) found that serum levels of Tau are significantly higher in jaundiced term newborns that manifest auditory neuropathy and neurologic abnormalities than those without these abnormalities, suggesting that axonal injury by exposure to UCB may lead to Tau release into the extracellular space [86].

CHAPTER 2

AIM

2. AIM

The main goal of the present project is to shed some light on the effect of UCB on cytoskeletal dynamics. As a first step to address this question we sought to dissect the mechanisms that underlie the changes of axonal outgrowth induced by UCB, focusing on: (i) the expression and localization of cytoskeleton associated proteins, namely the microtubule EB3, as an indicator of microtubule dynamic formation in maturating neurons; (ii) the microtubule stability, by treating neurons with vinblastine, a microtubule destabilizing agent; (iii) the expression, localization and function of cytoskeleton stabilizing molecules, such as MAP2 or Tau-1, which specifically stabilizes the dendrites or the axon, respectively; (iv) the localization and function of motor proteins, including dynein and kinesin, which are involved in the transport of cytoplasmic material along the axon; and on (v) the presence of viable mitochondria along the axon as an indicator of the functionality of axonal transport and of the energetic status of the cell.

CHAPTER 3

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. MATERIAL

For cell cultures neurobasal media, B27 supplement, Hanks' Balanced Salt Solution (HBSS), and Tripsin 2.5% were purchased from Gibco® (Grand Island, NY). Poly-D-lysine, Hoechst dye 33258, human serum albumin (HSA) and bovine serum albumin (BSA) (fraction V, fatty acid free) and UCB, that was purified according to McDonagh and Assisi method [87] and was always handled under light protection to avoid photodegradation, was acquired from Sigma-Aldrich. Laminin was from Invitrogen™ (Carlsbad, CA, USA). Minimum essential medium (MEM) with Earle's salts, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer, sodium pyruvate, fetal bovine serum (FBS) and glutamine were purchased from Biochrom AG (Berlin, Germany). For protein quantification assays it was used the protein assay kit, BioRad, (Hercules, California, CA). Sodium dodecyl sulphate (SDS) was acquired to VWR-Prolabo. Acrylamide, bis-acrylamide and Tween 20 were from Merck (Darmstadt, Germany). Nitrocellulose membrane and Hyperfilm ECL were from Amersham Biosciences (Piscataway, NJ, USA. LumiGLO® was from Cell Signalling (Beverly, MA, USA). Primary specific monoclonal antibodies were mouse anti-Tau1, mouse and rabbit anti-Map2, mouse anti-Kinesin and anti-Dynein from Chemicon® and mouse anti-β-actin from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies were Horseradish peroxidise-labeled goat anti-mouse IgG from Santa Cruz Biotechnology® (Santa Cruz, CA, USA) and horse anti-mouse from Vector Laboratories (Burlingame, CA). MitoTracker® Red m7512 was from Molecular Probes.

3.2. ANIMALS

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. Every effort was made to minimize the number of animals used and their suffering.

3.3. HIPPOCAMPAL NEURONAL CELL CULTURE

Primary culture of hippocampal neurons was prepared from hippocampi of E16 mice as previously described [88]. Shortly, pregnant mice were euthanized by asphyxiation with CO_2. Fetuses were removed from uterus and decapitated; heads were placed in a Petri dish with HBSS solution. Brain was carefully removed from head, cerebral hemispheres were dissected and meninges were removed. Finally, hippocampi were dissected, placed in a

microcentrifuge tube with HBSS solution and dissociated with 2.5% trypsin at 37ºC, followed by mechanical dissociation with a Pasteur pipette. For immunocytochemical studies approximately 2 x 10^4 cells were plated on each 12 mm coverslip, while for western blot analysis cells were plated with a density of approximately 2 x 10^5 cells per petri dish of 35mm. Both coverslips and petri dishes were coated with poly-D-lysine (100 µg/ml) and laminin (4 µg/ml) in plating medium (MEM with Earle's salts supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 0.5 mM glutamine, 12.5 µM glutamate, 10% FCS and 0.6% glucose). Three hours after, the media was replaced with neuronal growth medium (Neurobasal media with B27 supplement and 0.5 mM glutamine). At 1 day *in vitro* (DIV), cells were incubated with 50 or 100 µM UCB or with vehicle (culture medium plus 100 µM HSA to keep UCB in solution), for 24 h, at 37ºC. Following the UCB treatment, the incubation media was replaced by conditioned media from a parallel set of culture dishes with neurons at the same state of differentiation and cells were analyzed at 3 DIV (Fig.5).

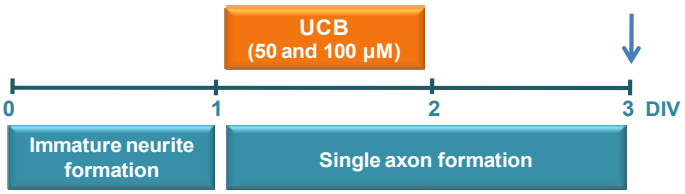


Fig.5. Schematic representation of the experimental design with primary hippocampal neurons. Cells were plated onto poly-D-lysine and laminin-coated coverslips and incubated at 1 day *in vitro* (DIV) with 100 µM HSA (vehicle) or UCB at 50 and 100 µM for 24 h at 37ºC. After UCB incubation, media was replaced with conditioned neuronal growth media without UCB and neurons were analyzed at 3 DIV.

3.4. ANALYSIS OF AXONAL LENGTH

Axon length measurements were performed on stage 3 neurons. For the purpose of this analysis, a neuron was defined as stage 3 if it had a single neurite (the axon) that was at least twice as long as all the other neurites. At 3 DIVs cells were fixed with PPS [(60 mM PIPES, pH 7.0; 25 mM HEPES, pH 7.0; 10 mM EGTA, pH 8.0; 2 mM $MgCl_2$; 0.12 M sucrose; 4% paraformaldehyde), (pH 7.3-7.4)] for 30 min at room temperature (RT). After rinsing in PBS, coverslips were blocked in 3% fatty acid free BSA in PBS for 30 min, permeabilized for 10 min in 0.2% triton/PBS, rinsed, and reblocked in 3% BSA/PBS for 30 min. Incubations with primary and secondary antisera were done in the presence of 1% BSA/PBS, and coverslips mounted with glycerol-based mountant (2.5% 1,4-Diazabicyclo-[2.2.2]Octane, 150 mM Tris (pH 8.0), 30% glycerol) to reduce photo bleaching. Images were captured on an Axiocam HR adapted to an Axiovert Microscope (Zeiss) using Openlab software (Improvision). The primary antibodies used were rabbit anti-MAP2 (1:1000, Chemicon) to

identify dendrites and mouse anti-Tau1 (1:200, Chemicon) to identify the axon. Axons were manually traced and their length measured using ImageJ v1.40.

3.5. EVALUATION OF MICROTUBULE FORMATION

To assess the rate of microtubule formation, neurons were electroporated prior to seeding with a plasmid expressing EGFP-tagged EB3 under a β-actin promoter (pCAG-EGFP-EB3) generated at Lorene M Lanier's laboratory (5 µg of plasmid for 1 x 10^6 cells) using the Mouse Neuron Nucleofector kit (Amaxa). After treatment, cells were fixed at 3 DIV with PPS, 30 min at RT, and EB3 staining was detected using a fluorescent microscope. The location of the microtubule EB3-positive tips was measure as the fluorescence intensity present along the axon, using ImageJ v1.40, to express the rate of microtubule polymerization.

3.6. APPRAISAL OF MICROTUBULE STABILITY

To evaluate microtubule stability, neurons already exposed to UCB were treated with 0.5 µM vinblastin, a microtubule destabilizer, for 2 h prior to cell fixation at 3 DIV. The ability of vinblastin to depolimerize the microtubules, indicating their less stabilization, was determined by the analysis of axonal length performed in fixed cultures stained with mouse anti-tubulin βIII (1:1000, Promega) as previously described.

3.7. DETERMINATION AND QUANTIFICATION OF MAP2 AND TAU1 EXPRESSION ALONG THE AXON AND THEIR BINDING TO MICROTUBULES

For the localization of MAP2 and Tau1, cells were treated as previously described (3.4.) and fluorescence intensity measured along the axon, using ImageJ v1.40. To determine the amount of MAP2 and Tau1 that was bound to the microtubules, cells were subjected to a saponin extraction prior to fixation. In brief, cells were exposed to 0.02% (w/v) saponin in extraction buffer (80 mM PIPES/KOH, ph 6.8; 1 mM $MgCl_2$; 1 mM EGTA; 30% (v/v) glycerol; 1 mM GTP)] for 30 sec at RT, to remove soluble cytoplasmatic proteins, and then fixed and treated as abovementioned.

3.9. DETERMINATION OF MOTOR PROTEINS KINESIN AND DYNEIN EXPRESSION AND LOCATION

In order to address kinesin and dynein expression and location, cells were treated as previously described (3.4) but immunostained with specific antibodies directed to these two proteins (1:100 for both, Chemicon) and fluorescence intensity was measured along the axon, using ImageJ v1.40.

3.10. DETECTION OF VIABLE MITOCHONDRIA WITH MITOTRACKER®

To evaluate mitochondria viability and localization, 3 DIV hippocampal neurons were incubated with MitoTracker® Red CMXRos (1:2000), which is a mitochondrion-selective dye that accumulates in active mitochondria, in growth medium for 30 min at 37ºC, always protected from light. Then cells were fixed with PPS for 30 minutes, at 37ºC. After rinsing with PBS, neurons were blocked with 3% BSA overnight at 4ºC. To identify axonal structure it was performed an immunofluorescence assay directed to Tau1 as described above. Nuclei were identified with Hoechst dye 33258 (1:1000), for 2 min at RT. The location of viable mitochondria was measure as the fluorescence intensity present along the axon, using ImageJ v1.40.

3.11. STATISTICAL ANALYSIS

Data are presented as means ± SEM, and statistical significance was determined by student's T-test, considering $P<0.05$ as statistically significant. On each experiment between 40 and 50 neurons were sampled per condition and each experiment was repeated at least three times.

CHAPTER 4

RESULTS

4. RESULTS

4.1. TREATMENT OF IMMATURE HIPPOCAMPAL NEURONS WITH UNCONJUGATED BILIRUBIN IMPAIRS AXONAL ELONGATION

Hippocampal neurons develop in a characteristic manner and acquire their polarized morphology in five well-defined stages [89], which may be altered upon injury. To characterize the effect of UCB on axonal elongation, 3 DIV neurons treated with vehicle, UCB 50 μM and UCB 100 μM were immunostained with MAP2 and Tau1 to identify dendrites and axon, respectively, and axonal length was analyzed. UCB exposure led to a reduction in axonal length in a concentration dependent manner. As observed in Figure 6A, vehicle-treated neurons present an increased percentage of neurons with an axonal length above 100 μM (~30%) than the ones exposed to UCB (~20%). When the average length of the axons was calculated (Fig. 6B), we observed that UCB treatment significantly reduced axonal outgrowth (~10%, $p<0.05$, and ~15%, $p<0.01$, for UCB 50 and 100 μM, respectively). These data are in accordance with a previous study from our group [59] that describes a reduction on axonal length and arborization, suggesting a negative effect of UCB on the formation of the neuronal network.

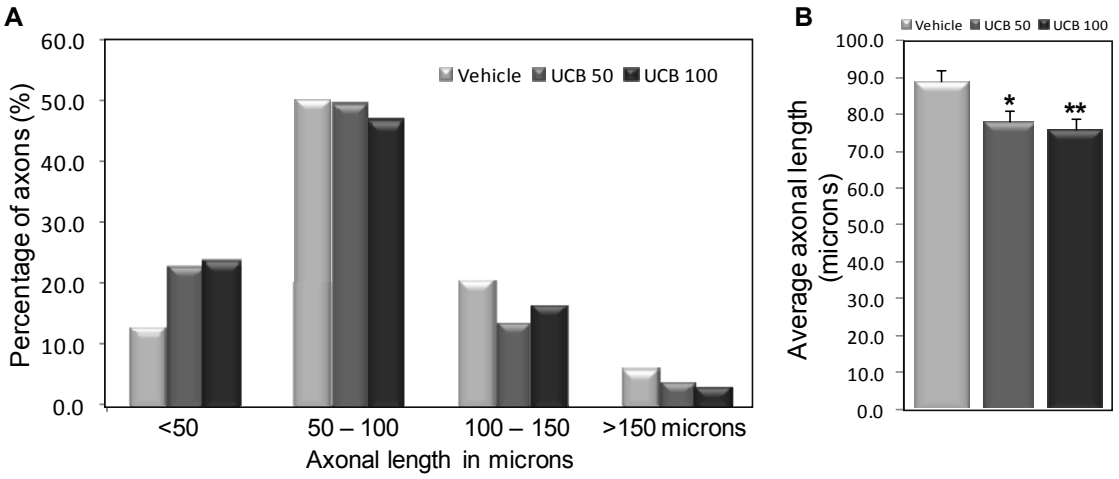


Fig.6. Bilirubin impairs axonal elongation. Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV and fixed and stained for MAP2 and Tau1 at 3 DIV. Cells were visualized by fluorescence, axons were manually traced and axonal length measured using ImageJ v1.40 and the NeuronJ plugin v1.4.0. (A) The percentage of axons presenting different lengths; (B) Average of axonal length for the different treatments. Results are at least from 3 independent experiments. *$p<0.05$ and **$p<0.01$ *vs.* vehicle.

4.2. EXPOSURE OF IMMATURE HIPPOCAMPAL NEURONS TO UNCONJUGATED BILIRUBIN REDUCES MICROTUBULES POLYMERIZATION AT THE APICAL PORTION OF THE AXON.

Cytoskeletal dynamics plays an essential role on neuronal morphogenesis [8]. Indeed, the axon formation requires the polymerization of microtubules and these cytoskeletal changes are regulated by microtubule-plus-end associated proteins as EB3, which recognize newly polymerized microtubules and provide an interface for interaction of microtubules array with actin cytoskeleton [33]. To assess the effect of UCB on the rate of microtubule formation, immature hippocampal neurons were transfected prior to seeding with a plasmid expressing EGFP-tagged EB3, incubated with vehicle, UCB 50 μM and UCB 100 μM for 24 h at 1 DIV and fixed at 3 DIV. Analysis of EB3 fluorescence along the axon (Fig. 7) showed that UCB significantly reduces the presence of EB3, with a more marked effect at the apical portion of the axon (~30% and ~25%, $p<0.05$ for UCB 50 and 100 μM, respectively), pointing to a deficient polymerization of microtubules at this structural level.

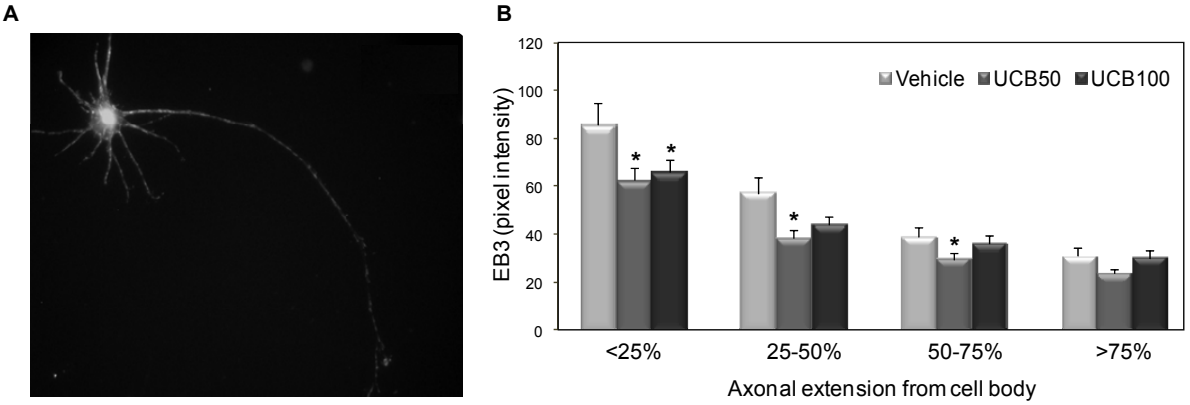


Fig.7. Unconjugated bilirubin reduces microtubules polymerization rate in immature hippocampal neurons. Embryonic hippocampal neurons expressing EGFP-tagged EB3 were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV and fixed at 3 DIV. Cells were visualized by fluorescence and EB3 fluorescence measured using the NeuronJ plugin v1.4.0. (A) Representative image of a hippocampal neuron expressing EB3 (in white). Original magnification: 200x. (B) Graph bars represent the EB3 pixel intensity (mean ± SEM) along the axon at 3 DIV. *$p<0.05$ *vs.* vehicle.

4.3. UNCONJUGATED BILIRUBIN INCREASES MICROTUBULES STABILITY OF IMMATURE HIPPOCAMPAL NEURONS CYTOSKELETON

Although, neuronal polarization and axon formation correlates with increased microtubules stability [16], axonal elongation needs a certain degree of microtubule instability, *dynamic instability*, for the maintenance of a polymerization-depolymerization cycle [18]. Thus, since we observed that both axonal outgrowth and microtubule polymerization are decreased

upon UCB exposure, we next evaluated the ability of UCB to alter microtubule stability. For that purpose neurons were incubated with vehicle medium, UCB 50 μM and 100 μM, at 1 DIV, a time-point where neurons are already polarized and axon is in an outgrowth stage, for 24 h. Then, at 3 DIV, the cells were incubated with vinblastin (0.5 μM), a potent microtubule destabilize [90], 2h prior to fixation. Then axonal extension from cell body was determined in neurons labeled for tubulin βIII. As shown in Figure 8, exposure of hippocampal cells to vinblastin led to a significant reduction of axonal length (~20%, $p<0.001$) as expected. Interestingly, neurons incubated with UCB, although showing a decreased axonal extension (~10%, $p<0.05$ and ~15%, $p<0.01$ for UCB 50 and 100 μM, respectively) when compared to vehicle-treated cells, presented no significant reduction of axonal length when incubated with vinblastine. These results suggest that in the presence of UCB, vinblastine cannot destabilize microtubules in the same way done in vehicle-treated cells, which points to an increased microtubule stability induced by UCB.

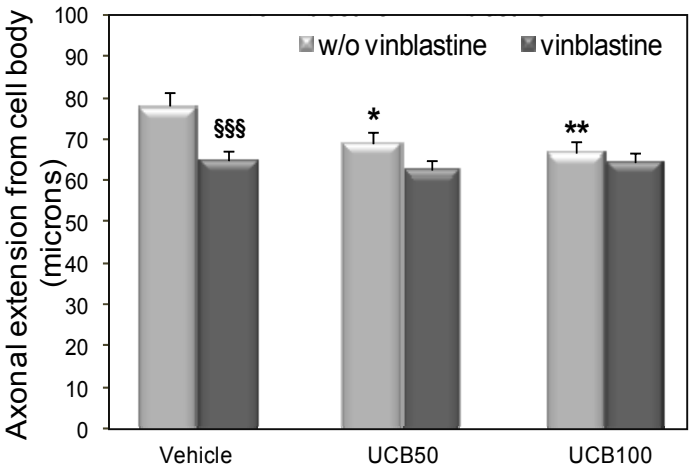


Fig.8. Exposure to unconjugated bilirubin increases microtubule stability in maturating neurons. Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV and exposed to vinblastine 0.5 μM at 3 DIV for 2 h prior to fixation. Cells were immunostained for tubulin βIII and visualized by fluorescence. Axons were manually traced and axonal length measured using ImageJ v1.40 and the NeuronJ plugin v1.4. *$p<0.05$ and **$p<0.01$ *vs*. vehicle. §§§$p<0.001$ *vs*. no vinblastine treatment.

4.4. UNCONJUGATED BILIRUBIN INDUCES CHANGES IN THE AXONAL LOCALIZATION PATTERN OF MAP2 AND TAU1 ON IMMATURE HIPPOCAMPAL NEURONS

The stabilization of microtubules is maintained by classical microtubule-associated proteins such as MAP2 and Tau1 [21]. Physiologically, MAP2 and Tau1 are differentially compartmentalized, with MAP2 localized at cell body and dendrites, whereas Tau1 is preferentially on the axon [89]. Hence we decided to evaluate whether UCB may affect MAP2 and Tau1 expression and localization. Primary hippocampal neurons were exposed to vehicle, UCB 50 μM and 100 μM, at 1 DIV for 24 h, fixed at 3 DIV and immunostained with

MAP2 and Tau1 antibodies. To address MAP2 and Tau1 microtubule binding, cytoplasmic fractions of these proteins were saponin extracted prior to fixation.

4.4.1. Bilirubin increases MAP2 axonal entry and enhances axonal MAP2 binding to microtubules

Exposure of immature neurons to UCB induced an increased axonal entry of MAP2 (Fig.9A). This effect was analyzed by measuring the percentage of the axonal length that presented MAP2 fluorescence intensity above 50% (Fig.9B). These data point out that MAP2 only reaches about 8% of the apical portion of the axon in vehicle treated cells, but upon UCB treatment MAP2 axonal entry is increased in a concentration-dependent manner reaching ~14 (p<0.01, UCB 50 μM) and ~ 16% (p<0.001, UCB 100 μM) of the axon. So, this effect may contribute to changes in microtubule organization and impairment of cytoskeleton function, since MAP2 has an important role on initially lamella-like structures formation during cell polarization [32,91].

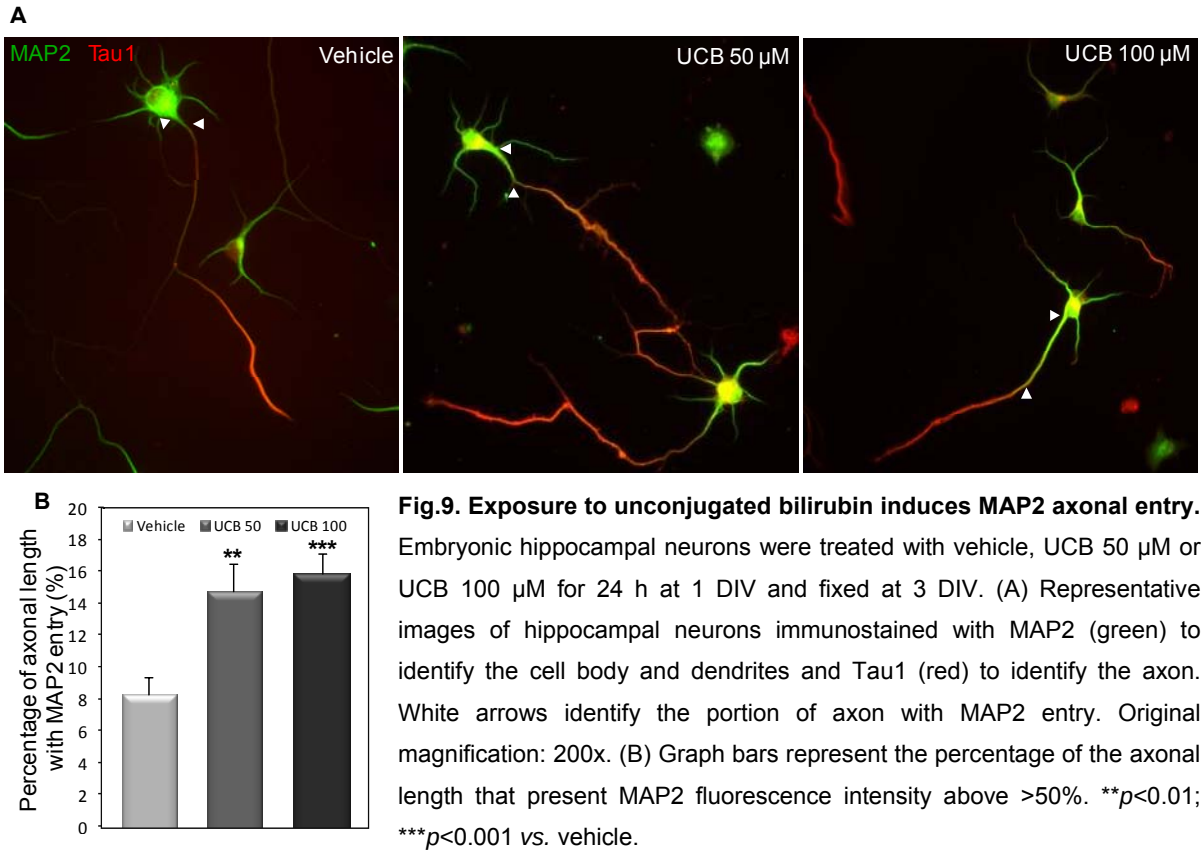


Fig.9. Exposure to unconjugated bilirubin induces MAP2 axonal entry. Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV and fixed at 3 DIV. (A) Representative images of hippocampal neurons immunostained with MAP2 (green) to identify the cell body and dendrites and Tau1 (red) to identify the axon. White arrows identify the portion of axon with MAP2 entry. Original magnification: 200x. (B) Graph bars represent the percentage of the axonal length that present MAP2 fluorescence intensity above >50%. **p<0.01; ***p<0.001 *vs.* vehicle.

The evaluation of MAP2 expression throughout the axon revealed that it was significantly increased by UCB exposure in a concentration dependent way (Fig. 10). When the total amount of MAP2 staining was analyzed (Fig. 10A), UCB showed to increase MAP2 expression mainly in the apical portion of the axon (~30%, p<0.05 and ~70%, p<0.001 for UCB 50 and 100 μM, respectively). After saponin extraction (Fig.10B), the remaining MAP2

that was bound to the microtubules appeared to be more enhanced by UCB in the middle region (~40%, p<0.001 for UCB 100 μM) and in the most distal parts of the axon (~25%, p<0.01 for UCB 50 μM).

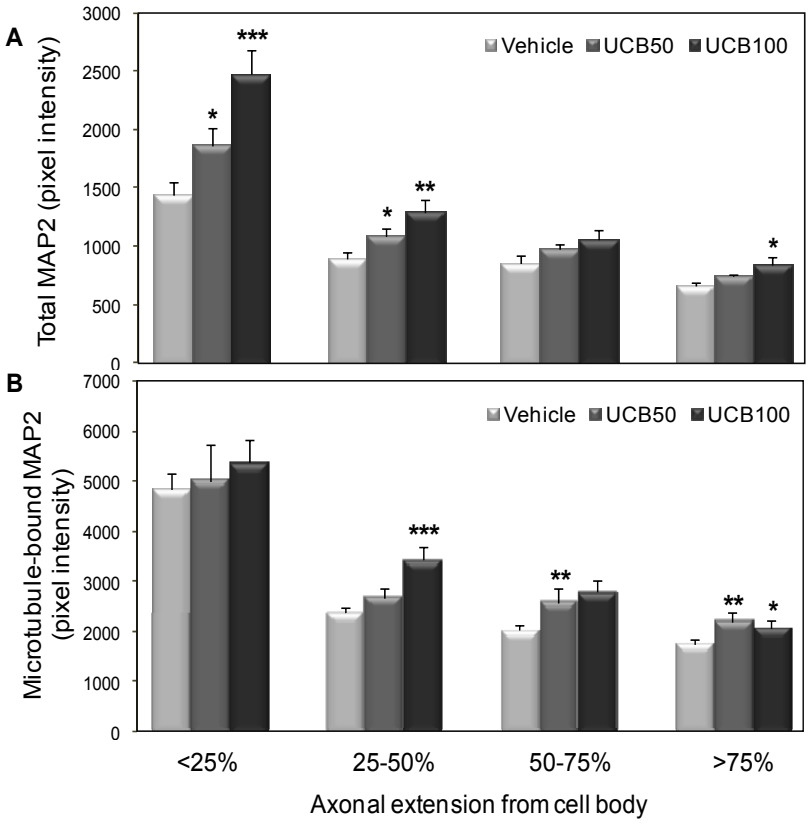


Fig.10. Treatment of immature hippocampal neurons with unconjugated bilirubin increases axonal expression of MAP2 and its binding to microtubules. Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV. At 3 DIVs, cells were either fixed or extracted with saponin prior to fixation in order to remove cytoplasm soluble compounds and therefore to reveal only cytoskeletal bound proteins. Following immunostaining for MAP2 axonal fluorescence intensity was measured using ImageJ v1.40. Graph bars represent total (A) or microtubule-bound (B) MAP2 expression along the axon. *p<0.05; **p<0.01; ***p<0.001 *vs.* vehicle.

4.4.2. Unconjugated bilirubin increases both axonal Tau1 expression and Tau1 binding to microtubules

Exposure of immature hippocampal neurons to UCB led to alterations on Tau1 expression levels. Concerning total Tau1 expression (Fig. 11A), UCB 50 μM significantly increased Tau1 pixel intensity (>60%, p<0.001), whereas UCB 100 μM decreased it (~20%, p<0.001) mainly at the middle portion of the axon. Interestingly, when only the microtubule-bound Tau1 was analyzed, we verified that both UCB concentrations increased Tau1 binding to microtubules, in a concentration-dependent manner all along the axonal length (~40%, p<0.01 and >80%, p<0.001 for UCB 50 μM and 100 μM, respectively). These data suggest that while in vehicle and mainly in 50 μM UCB-treated cells there is a great proportion of Tau1 protein that is soluble in the cytoplasm, while in 100 μM UCB-treated cells the amount of Tau-1 that is expressed appears to be almost exclusively bound to the microtubules.

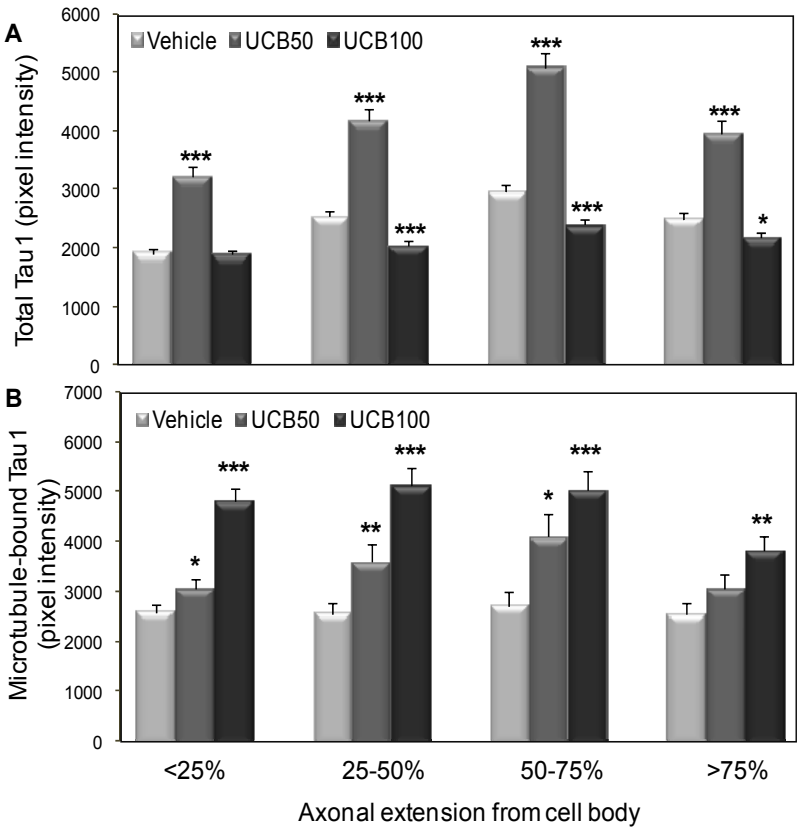


Fig.11. Unconjugated bilirubin alters Tau1 axonal expression and increases Tau1 binding to microtubules on immature hippocampal neurons.

Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV. At 3 DIVs, cells were either fixed or extracted with saponin prior to fixation to remove cytoplasm soluble compounds and to only reveal cytoskeletal bound proteins. Following immunostaining for Tau1 axonal fluorescence intensity was measured using ImageJ v1.40. Graph bars represent total (A) or microtubule-bound (B) Tau1 expression along the axon. **p*<0.05; ***p*<0.01; ****p*<0.001 *vs.* vehicle.

These data suggest that UCB induces significant alterations on the expression pattern of these two proteins, affecting their location, expression levels and also their binding capability to the microtubules. Indeed, UCB appears to interfere with neuronal cytoskeleton integrity, which may lead to loss of neuronal functionality, potentially impairing axonal transport.

4.5. TREATMENT OF IMMATURE HIPPOCAMPAL NEURONS WITH UNCONJUGATED BILIRUBIN INDUCES CHANGES ON AXONAL TRANSPORT-RELATED PROTEINS

Intracellular transport of protein and organelles is essential for cell function and organization, acquiring particular importance in neurons where cargoes must move for long distances through the axon [92]. Kinesin and dynein are motor proteins responsible for anterograde and retrograde transport, respectively. Most attractive is the fact that this normal performance can be affected by microtubule associated proteins as MAP2 and Tau1 [42]. Therefore, we next analyzed the expression and localization of kinesin and dynein in hippocampal neurons treated with vehicle, UCB 50 μM or 100 μM for 24 h at 1 DIV. As demonstrated in Figure 12, exposure to UCB changed the expression levels of both motor

proteins. Regarding kinesin, UCB reduced its expression all along the axon in a concentration dependent manner (Fig.12A), reaching significant values for the higher UCB concentration (~10%, $p<0.05$ for UCB 100 μM).On the other hand, when dynein expression was measured (Fig. 12B), the two concentrations of UCB used had contrary effects. Indeed, dynein expression was significantly decreased by UCB 50 μM (~10%, $p<0.05$), but highly enhanced by UCB 100 μM (~40%, $p<0.001$) along the axon.

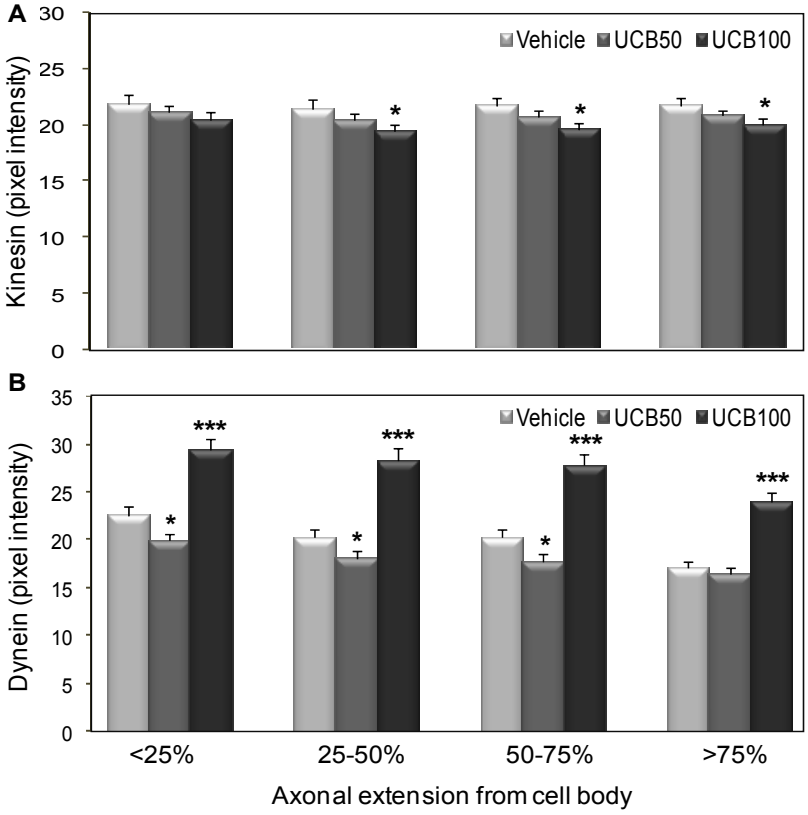


Fig.12. Unconjugated bilirubin induces changes on axonal transport-related proteins.
Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV. At 3 DIVs, cells were fixed, immunostained for kinesin and dynein and axonal fluorescence intensity was measured using ImageJ v1.40. Graph bars represent kinesin (A) or dynein (B) expression along the axon *$p<0.05$;***$p<0.001$ *vs*. vehicle.

These data suggest that axonal transport may be disturbed by UCB since treated neurons present changes in the motor proteins expression pattern, which may impair the balance between anterograde and retrograde transport.

4.6. UNCONJUGATED BILIRUBIN INCREASES MITOCHONDRIA AGGREGATION AT THE DISTAL PORTION OF THE AXON

Mitochondria are dynamic organelles that are essential for neuronal function and survival, and one of the many cargoes transported by motor proteins as kinesin and dynein [43,93]. Thus, we decided to next evaluate whether an exposure to UCB would also alter mitochondria localization and viability along the axon. Following treatment with vehicle or with UCB at 50 μM or 100 μM for 24 h at 1 DIV, at 3 DIV cells were exposed to MitoTracker®, a compound that integrates the membrane of viable mitochondria. Although

we were unable to find any relevant differences on mitochondria pixel intensity along the axon when UCB-treated cells were compared with vehicle ones (data not shown), we observed that mitochondria was forming aggregates in certain regions of the axons. Therefore, we decided to count the mitochondria clusters along the axon, defining a cluster as an aggregate of mitochondria presenting a diameter above 2 μm. As shown in Figure 13, vehicle-treated neurons usually present an increased accumulation of mitochondria cluster in the apical part of the axon. Interestingly, incubation with UCB shifted the mitochondria aggregates to a more medial and distal part of the axon. Indeed, while for a lower concentration of UCB the mitochondria clusters are mostly augmented in the middle and in the distal regions of the axon (~25%, $p<0.05$), the higher concentration of UCB mainly increased mitochondria aggregates at the distal portion of the axon (>55%, $p<0.05$). These data point to an impaired distribution of mitochondria on the axonal compartment of hippocampal immature neurons following UCB exposure.

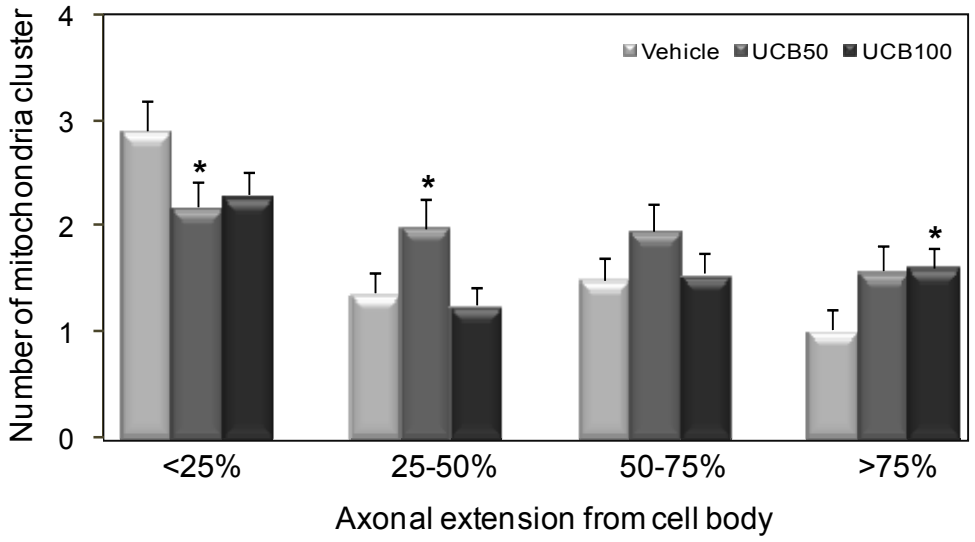
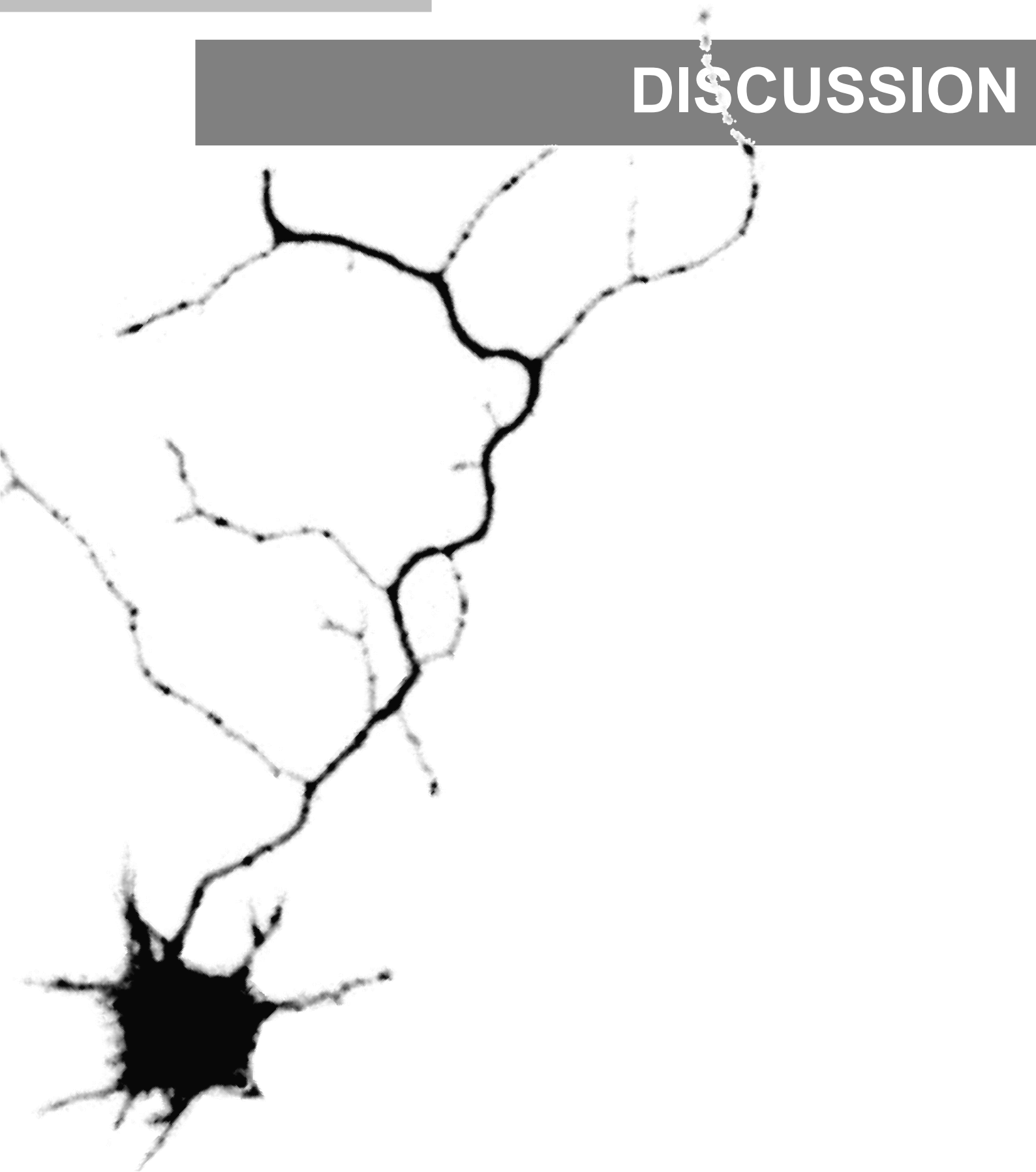


Fig.13. Bilirubin increases the aggregation of mitochondria at the median and distal portion of the axon. Embryonic hippocampal neurons were treated with vehicle, UCB 50 μM or UCB 100 μM for 24 h at 1 DIV. At 3 DIVs, cells were incubated with MitoTracker®, a compound that integrates the membrane of viable mitochondria, and fixed. Graph bars represent the number of mitochondria cluster (aggregates >2 μm) counting through the axon (average ± SEM). *$p<0.05$ *vs.* vehicle.

CHAPTER 5

DISCUSSION



5. DISCUSSION

Hyperbilirubinemia, one of the most common conditions in the neonatal period, may under certain circumstances induce reversible neurological injury [49] and has more recently been associated with the onset of schizophrenia [52]. In this context, we and others have already demonstrated in both *in vitro* and *in vivo* studies that UCB has numerous neurotoxic effects, acting in several cellular mechanisms [69,82] and compromising cellular viability [58,94]. Interestingly, it was also reported that UCB is able to impair neuronal synaptic signaling [82-84], and more recently, we have shown that morphologically UCB reduces neurite arborization, dendritic spine density and synaptic formation in developing neurons [59].

Since the elaboration of neural architecture is dependent on a functional cytoskeleton, in the present study we have further dissected the effects of UCB on neuronal cytoskeleton dynamics, namely at the axonal level. Here, we present evidence that exposure of immature hippocampal neurons to UCB induce modifications on neuronal cytoskeleton by (i) impairing axonal elongation and microtubule polymerization; (ii) increasing microtubule stability and therefore decreasing the *dynamic instability* needed for axonal elongation; (iii) changing microtubule associated proteins, MAP2 and Tau1, expression and localization patterns; (iv) damaging axonal transport by decreasing kinesin and increasing dynein expression along the axon and (v) inducing mitochondria to aggregate and accumulate on the distal portion of the axon.

We have observed that UCB impairs axonal elongation of hippocampal neurons, with an effective reduction of the average axonal length and an increase of the number of shorter axons, suggesting that UCB acts on the axonal formation event. These data are in accordance with those described in our previously published work [59]. Although cerebellar hypoplasia and reduction of the number of Purkinje cells have been identified in an animal model of *kernicterus* [95] and a recent work described a wide spectrum of magnetic resonance imaging (MRI) abnormalities in preterm and term infants with *kernicterus* [96], to our knowledge no data regarding the UCB effect on *in vivo* neurite outgrowth have been to now described. Interestingly, MRI studies suggested excessive elimination of dendrites and axons of prefrontal cortex of schizophrenics with significant volume loss of temporal cortex and frontal lobe [97,98].

Microtubules array have crucial roles on cell morphology and polarization. Key events, as the separation of the *lamellipodium* from the cell soma to become a growth cone or axonal elongation, are dependent on both the presence and the dynamic properties of microtubules[12]. Our data demonstrate that UCB interferes with normal microtubule polymerization, observed by a reduction of EB3 expression. Since microtubule polymerization contributes to microtubules array establishment [99], our data suggest that

neonatal hyperbilirubinemia impairs microtubule architecture possibly leading to deficient axonal growth, which may have repercussion on neuronal network and communication. Accordingly, it was described that in the absence of normal microtubule polymerization, processes fail to form growth cones and to generate a thin neurite shaft [12]. Curiously, when the images of neurons expressing EGFP-tagged EB3 are observed in detail it seems that UCB-treated neurons present a thicker apical axonal portion with less bundle microtubules (data not shown).

Although microtubule stabilization has an active function in the specification of neuronal polarization and axonal fate in early neuronal development [16], during axonal elongation microtubule stability is tightly regulated by endogenous microtubule destabilizing factors which maintain a crucial polymerization-depolymerization cycle referred often as a microtubule *dynamic instability* [18]. Our results show that exposure of polarized neurons to UCB stabilizes neuronal microtubules in a concentration dependent manner, preventing their depolarization upon vinblastine treatment, which is a potent destabilizing agent [100]. Based on recent studies showing that neurite extension and growth cone motility are reduced with the loss of superior cervical ganglia 10 (SCG10), an endogenous destabilizing protein [18]. It seems that UCB reduces axonal outgrowth by increasing axonal microtubule stability. However, others showed that downregulation of another destabilizing protein, SCG10-like protein (SCLIP), enhances axonal branching [101], which is also impaired upon UCB treatment as previously demonstrated [59]. Curiously, some authors describe that microtubules stabilizing drugs such as paclitaxel have therapeutic potential for treating neurodegenerative tauopathies [102] or to induce axonal regeneration in CNS lesioning situations [16]. On the other hand, that the use of this same drug was reported to be toxic to mature neurons in the hippocampus in vivo [103]. This contradictory information reveals that microtubules dynamic mechanisms must be crucially regulated in a time-dependent manner along neuronal development either under normal conditions or following a neurological disease. Additional studies evaluating the expression of tyrosinated and acetylated tubulin, that reflect newly assembled and mature stable microtubules, respectively[104], are underway in our laboratory to better define the UCB effect at this level.

Microtubules associated proteins MAP2 and Tau1 have essential and distinct roles in the early stages of neuromorphogenesis, while the initial establishment of neurites depends upon MAP2, further neurite elongation depends upon Tau1 [30]. Indeed, antisense depletion of MAP2 was shown to inhibit neurite initiation [30], while Tau antisense suppression was reported to prevent axon initiation in cultured cerebellar macroneurons [105]. Accordingly to the increased microtubule stability observed in neurons exposed to UCB, UCB also increased the expression of microtubule associated proteins, as well as their binding to microtubules. We showed that UCB increases MAP2 entry in the axonal compartment. Since MAP2 has a

preponderant role on neurite initiation, we can hypothesize that UCB retards the neuronal differentiation, affecting neuronal polarization and axonal formation, which is in accordance with our previous work arguing that UCB promotes alterations on neuritogenesis [59]. Our data also show that UCB promotes increased MAP2 binding to microtubules, which has been reported to increase microtubule rigidity [106] and consequently to impair their dynamic action to answer to morphological and functional needs of the cell cytoskeleton. Fascinatingly, stimulation of MAP2 expression on hippocampal region has been related with ischemic injury[107], structural changes induced by estradiol and progesterone [108], kainic acid-induced status epilepticus [109] and in motoneurons from rats with acute spinal cord injury [110]. This points to a relevant role of MAP2 on the mechanisms developed on cellular stress situations.

Furthermore, our data also demonstrate an increased expression of Tau1 induced by moderate hyperbilirubinemia, while a most severe condition leads to decreased Tau1 expression. Intriguingly, when we look to Tau1 binding to microtubules, we find exactly the opposing effect. Here, both UCB concentrations increased Tau1 binding to microtubules in a concentration-dependent manner, suggesting that, despite the decreased Tau1 expression observed for UCB 100 μM, the existing protein is mostly bound to microtubules. Moreover, we may also hypothesize that the most severe condition of hyperbilirubinemia can potentiate Tau1 release to the extracellular space, and so, we are only able to detect the remaining Tau1 that is bound to the cytoskeleton. Interestingly, a recent study reported that jaundiced term newborns that manifested auditory neuropathy, neurologic abnormalities or electroencephalogram abnormalities have significantly higher serum Tau levels [86]. Sustained loss of Tau from axonal microtubules over time renders them more sensitive to endogenous severing proteins, thus causing the microtubule array to gradually disintegrate. This condition is reported as the basis for neuronal degeneration in tauopathies such as Alzheimer's disease [27].

Recently, it was shown that levels of total-Tau and of phosphorylated-Tau in cerebral spinal fluid are strongly associated with future development of Alzheimer's disease in patients with mild cognitive impairment [111,112]. In a more mechanistic approach, it has been described that hyperphosphorylated Tau sequesters normal MAPs, disrupts microtubule dynamics, blocks intracellular trafficking of the neurons and facilitates tau aggregation [26]. In addition, progressive morbidity with age is accompanied by increasing hyperphosphorylation of Tau [113]. In this regard, it would be relevant evaluate the phosphorylation status of Tau1 as a future approach to address the eventual effect of UCB on Tau1 post-translation modifications.

In vivo, the MAP2 and Tau projection domains appear to be involved in regulating microtubule spacing. Such control over microtubule spacing might facilitate efficient organelle transport [29]. Tau has been described to interfere with normal function of motor proteins,

reducing the reattachment rates, affecting cargo travel distance, motive force, and cargo dispersal, obviously impairing axonal transport [114]. In this regard, not only UCB increased Tau1 expression and binding to microtubules of hippocampal immature neurons, but also it was able to decrease kinesin expression and to increase dynein expression along the axon, suggesting an impairment of the anterograde axonal transport and an alteration in the retrograde axonal transport rate, respectively. Accordingly with our findings, a recent study described a decrease of anterograde transport motor proteins, as kinesin superfamilly (KIF) (KIF1A, KIF1B, KIF2A, and KIF3A) in parallel with an increase of retrograde motor proteins (dynein, dynamitin, and dynactin1) during striatal neuroinflammation in an animal model of α-synucleinopathy [115]. Most attractive is the fact that this phenomenon was associated with changes in the proteins involved in synaptic transmission and precedes neuronal loss. Hence, these early events induced by UCB may be important indicators of future neuronal demise.

In addition, it is known that Tau1 binding to microtubules can differently modulate kinesin and dynein motility. Whereas kinesin tend to detach at patches of microtubule bound Tau, dynein tends to reverse its direction[42]. This differential modulation also depends on Tau1 isoforms and its phosphorylation status[116,117]. Furthermore, dynein is less sensitive to Tau1 than kinesin, being able to attach to microtubules with higher levels of Tau1 on the contrary to kinesin [116]. Dynein has other abilities to proceed with its movement when encounters a patch of Tau1 such as to reverse its course, switch to an alternative microtubule [118], or give larger steps to bypass the blockage induced by Tau1 [119]. This can justify the elevated amount of dynein found in our cells exposed to UCB. As dynein plays a critical role on the neuronal response to injury and is able to transport important information from axonal synapses back to the nucleus [120], it would be relevant to investigate the exact mechanism of UCB action, namely whether these motor proteins are still functional and if their axonal transport is impaired or reversed. Injury of axonal transport is often described in many neurodegenerative diseases (for review see De Vos *et al.*, 2008). In this context, our results may suggest that the alterations induced by UCB exposure during neonatal period may impair vital neuronal functions that can be related with the development of neurodegenerative conditions in later life [92].

Mitochondria are really dynamic and plastic organelles. To maintain their mobility, mitochondria seem often associated to the microtubule network. In neuronal axons they can change between anterograde and retrograde movement, if they must go to the synaptic bouton or to the cell body for degradation and recycling, respectively [7,45]. Since UCB impairs axonal transport in immature hippocampal neurons, it would be expectable that mitochondria distribution will be also altered. Indeed, our data reveal that UCB increases mitochondria aggregation along the axon with a greater accumulation on the distal portion. A recent work

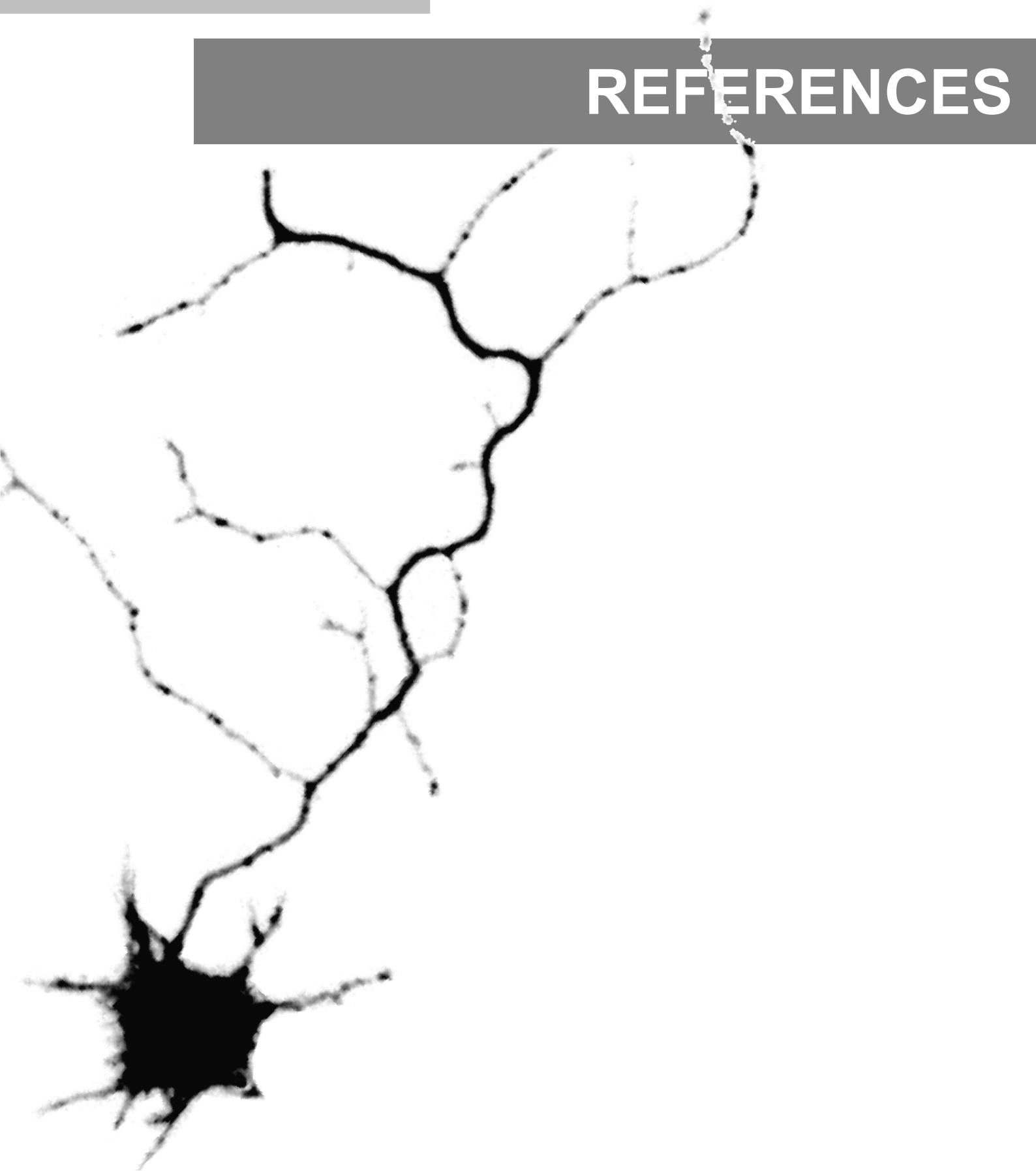
defends that mitochondria tend to fuse in response to stress, when exposed to different toxic agents [44] and mitochondrial morphological abnormalities in synaptic terminals and in distal regions of axons were identified in paraplegin-deficient mice before the first signs of degeneration [121]. In fact, mitochondria damaging mechanisms are described in many neurodegenerative conditions and age-related diseases [43,92].

Mitochondrial transport toward the axon is processed by two kinesin, namely, KIF1Ba and KIF5 [93], while its retrograde transport is only carried by dynein [122]. Given the higher levels of dynein induced by UCB, it would be expectable that mitochondria did not accumulate in the axon, but near to the cell body. Hence, we can speculate that the dynein present on the axon may not be functional, or cannot properly bind to clustered mitochondria. Further studies should evaluate the mitochondria movement along the axon using time lapse imaging to find some new insights about mitochondria viability and transport.

Collectively, our results demonstrate that exposure of immature hippocampal neurons to UCB induce modifications on neuronal cytoskeleton by impairing axonal elongation and microtubule polymerization, by increasing microtubule stability and by changing expression and localization patterns of the microtubule associated proteins MAP2 and Tau1, UCB also potentially acts on axonal transport by decreasing kinesin and increasing dynein expression along the axon. Moreover, UCB induce mitochondria aggregation and accumulation on the distal portion of the axon. Finally, because most of these events were described in several neurological and neurodegenerative diseases, the present work provide a basis for the gating mechanisms underlying the casual relationship between early brain damage by moderate and severe levels of jaundice and learning and memory deficits or neuropathological states later in life.

CHAPTER 6

REFERENCES



6. REFERENCES

1. Dale Purves, George J. Augustine, David Fitzpatrick et al., *Neuroscience*, Third ed. (Sinauer Associates, Inc, Massachusetts U.S.A. , 2004).
2. T. Yoshimura, N. Arimura, and K. Kaibuchi, *J Neurosci* **26** (42), 10626 (2006).
3. R.L. Squire, D. Berg, F.E. Bloom et al., *Fundamental Neuroscience*, 3rd ed. (Elsevier Inc., 2008).
4. D. Rice and S. Barone, Jr., *Environ Health Perspect* **108 Suppl 3**, 511 (2000).
5. P. J. Harrison, *Psychopharmacology (Berl)* **174** (1), 151 (2004).
6. R. Brandt, *Cell Tissue Res* **292** (2), 181 (1998).
7. B Alberts, A. Johnson, J. Lewis et al., *Molecular Biology of the Cell*, 5th ed. (Garland Science, 2008).
8. F. Bradke and C. G. Dotti, *Curr Opin Neurobiol* **10** (5), 574 (2000).
9. P. W. Baas, *Nat Cell Biol* **4** (8), E194 (2002).
10. F. Bradke and C. G. Dotti, *Microsc Res Tech* **48** (1), 3 (2000).
11. P. W. Baas, M. M. Black, and G. A. Banker, *J Cell Biol* **109** (6 Pt 1), 3085 (1989).
12. L. Dehmelt and S. Halpain, *J Neurobiol* **58** (1), 18 (2004).
13. P. M. Medina, R. J. Worthen, L. J. Forsberg et al., *PLoS ONE* **3** (8), e3054 (2008).
14. M. P. Mattson, *J Neurosci Res* **57** (5), 577 (1999).
15. D. J. Sharp, G. C. Rogers, and J. M. Scholey, *Biochim Biophys Acta* **1496** (1), 128 (2000).
16. H. Witte, D. Neukirchen, and F. Bradke, *J Cell Biol* **180** (3), 619 (2008).
17. S. E. Siegrist and C. Q. Doe, *Genes Dev* **21** (5), 483 (2007).
18. H. Morii, Y. Shiraishi-Yamaguchi, and N. Mori, *J Neurobiol* **66** (10), 1101 (2006).
19. S. Gomis-Ruth, C. J. Wierenga, and F. Bradke, *Curr Biol* **18** (13), 992 (2008).
20. H. Witte and F. Bradke, *Curr Opin Neurobiol* **18** (5), 479 (2008).
21. V. Fontaine-Lenoir, B. Chambraud, A. Fellous et al., *Proc Natl Acad Sci U S A* **103** (12), 4711 (2006).
22. M. Goedert, R. A. Crowther, and C. C. Garner, *Trends Neurosci* **14** (5), 193 (1991).
23. J. Ferralli, T. Doll, and A. Matus, *J Cell Sci* **107 (Pt 11)**, 3115 (1994).
24. R. L. Neve, P. Harris, K. S. Kosik et al., *Brain Res* **387** (3), 271 (1986).
25. J. F. Leterrier, M. Kurachi, T. Tashiro et al., *Eur Biophys J* **38** (4), 381 (2009).
26. J. Z. Wang and F. Liu, *Prog Neurobiol* **85** (2), 148 (2008).
27. L. Qiang, W. Yu, A. Andreadis et al., *J Neurosci* **26** (12), 3120 (2006).
28. D. N. Drechsel, A. A. Hyman, M. H. Cobb et al., *Mol Biol Cell* **3** (10), 1141 (1992).
29. L. Dehmelt and S. Halpain, *Genome Biol* **6** (1), 204 (2005).
30. A. Caceres, J. Mautino, and K. S. Kosik, *Neuron* **9** (4), 607 (1992).
31. S. Konzack, E. Thies, A. Marx et al., *J Neurosci* **27** (37), 9916 (2007).
32. S. L. Kwei, A. Clement, A. Faissner et al., *Neuroreport* **9** (6), 1035 (1998).
33. J. Jaworski, C. C. Hoogenraad, and A. Akhmanova, *Int J Biochem Cell Biol* **40** (4), 619 (2008).
34. T Stepanova, J Slemmer, C C Hoogenraad et al., *J Neurosci* **23(7)**, 2655 (2003).
35. H. Nakagawa, K. Koyama, Y. Murata et al., *Oncogene* **19** (2), 210 (2000).
36. F. J. Ahmad, Y. He, K. A. Myers et al., *Traffic* **7** (5), 524 (2006).
37. L. S. Goldstein and Z. Yang, *Annu Rev Neurosci* **23**, 39 (2000).
38. B. W. Guzik and L. S. Goldstein, *Curr Opin Cell Biol* **16** (4), 443 (2004).
39. R Dixit, J L Ross, Y E Goldman et al., *Science* **319(5866)**, 1086 (2008).
40. L Dehmelt and S Halpain, *Genome Biol* **6(1)**, 204 (2005).
41. P. W. Baas, C. Vidya Nadar, and K. A. Myers, *Traffic* **7** (5), 490 (2006).
42. R. Dixit, J. L. Ross, Y. E. Goldman et al., *Science* **319** (5866), 1086 (2008).
43. D. C. Wallace, *Annu Rev Genet* **39**, 359 (2005).
44. A. M. van der Bliek, *EMBO J* **28** (11), 1533 (2009).
45. P. J. Hollenbeck, *Front Biosci* **1**, d91 (1996).
46. P. J. Hollenbeck and W. M. Saxton, *J Cell Sci* **118** (Pt 23), 5411 (2005).
47. Q. Cai and Z. H. Sheng, *Exp Neurol* (2009).
48. M. P. Mattson, M. Gleichmann, and A. Cheng, *Neuron* **60** (5), 748 (2008).
49. American Academy of Pediatrics, *Pediatrics* **114** (1), 297 (2004).
50. S. M. Shapiro, *Pediatr Neurol* **29** (5), 410 (2003).
51. K. A. Jangaard, D. B. Fell, L. Dodds et al., *Pediatrics* **122** (1), 119 (2008).
52. T. Miyaoka, H. Seno, M. Itoga et al., *J Clin Psychiatry* **61** (11), 868 (2000).
53. T. Miyaoka, H. Seno, T. Maeda et al., *J Clin Psychiatry* **61** (4), 299 (2000).
54. T. Miyaoka, H. Seno, M. Itoga et al., *Schizophr Res* **52** (3), 291 (2001).
55. T. Miyaoka, R. Yasukawa, T. Mihara et al., *Eur Psychiatry* **20** (4), 327 (2005).
56. A. S. Falcão, R. F. Silva, S. Pancadas et al., *J Neurosci Res* **85** (6), 1229 (2007).
57. W. Oh, J. E. Tyson, A. A. Fanaroff et al., *Pediatrics* **112** (4), 773 (2003).
58. A. S. Falcão, A. Fernandes, M. A. Brito et al., *Acta Neuropathol* **112** (1), 95 (2006).
59. Adelaide Fernandes, Ana Sofia Falcão, Elsa Abranches et al., *Developmental Neurobiology* (2009).
60. D. S. Seidman, S. Dollberg, D. K. Stevenson et al., *Arch Gynecol Obstet* **249** (3), 119 (1991).
61. P. A. Dennery, D. S. Seidman, and D. K. Stevenson, *N Engl J Med* **344** (8), 581 (2001).
62. R. P. Wennberg, C. E. Ahlfors, V. K. Bhutani et al., *Pediatrics* **117** (2), 474 (2006).

63. A. Fernandes D. Brites, A. S. Falcão, M. A. Brito and R F. M. Silva, in *Encyclopedia of Neuroscience*, edited by Nobutaka Hirokawa and Uwe Windhorst Marc D. Binder (Springer-Verlag GmbH Berlin Heidelberg 2009).
64. D. K. Stevenson, P. A. Dennery, and S. R. Hintz, *J Perinatol* **21 Suppl 1**, S21 (2001).
65. R. L. Watson, *Crit Care Nurs Clin North Am* **21** (1), 97 (2009).
66. T. W. Hansen, *Clin Perinatol* **29** (4), 765 (2002).
67. D. E. Baranano, M. Rao, C. D. Ferris et al., *Proc Natl Acad Sci U S A* **99** (25), 16093 (2002).
68. MA Brito, RFM Silva, and D Brites, in *New Trends in Brain Research* (Nova Science Publishers, Inc., Lisbon, 2006).
69. D. Brites, A. Fernandes, A. S. Falcao et al., *J Perinatol* **29 Suppl 1**, S8 (2009).
70. G. R. Gourley, *Adv Pediatr* **44**, 173 (1997).
71. C. Roger, V. Koziel, P. Vert et al., *Pediatr Res* **39** (1), 64 (1996).
72. J. J. Volpe, *Brain Dev* **19** (8), 519 (1997).
73. C. M. Rodrigues, S. Sola, R. Silva et al., *Mol Med* **6** (11), 936 (2000).
74. C. M. Rodrigues, S. Sola, and D. Brites, *Hepatology* **35** (5), 1186 (2002b).
75. C. M. Rodrigues, S. Sola, R. E. Castro et al., *J Lipid Res* **43** (6), 885 (2002a).
76. A. Fernandes, A. S. Falcao, R. F. Silva et al., *Eur J Neurosci* **25** (4), 1058 (2007).
77. M. A. Brito, A. I. Rosa, A. S. Falcao et al., *Neurobiol Dis* **29** (1), 30 (2008).
78. R. F. Silva, C. M. Rodrigues, and D. Brites, *Pediatr Res* **51** (4), 535 (2002).
79. C. M. Rodrigues, S. Sola, R. F. Silva et al., *Pediatr Res* **51** (1), 112 (2002).
80. Y. Amit and T. Brenner, *Exp Neurol* **121** (2), 248 (1993).
81. J. F. Watchko and M. J. Maisels, *Arch Dis Child Fetal Neonatal Ed* **88** (6), F455 (2003).
82. J. D. Ostrow, L. Pascolo, D. Brites et al., *Trends Mol Med* **10** (2), 65 (2004).
83. L. Zhang, W. Liu, A. K. Tanswell et al., *Pediatr Res* **53** (6), 939 (2003).
84. F. Y. Chang, C. C. Lee, C. C. Huang et al., *PLoS ONE* **4** (6), e5876 (2009).
85. M. Hayashida, T. Miyaoka, K. Tsuchie et al., *Prog Neuropsychopharmacol Biol Psychiatry* (2009).
86. N. Okumus, C. Turkyilmaz, E. E. Onal et al., *Pediatr Neurol* **39** (4), 245 (2008).
87. A. F. McDonagh and F. Assisi, *Biochem J* **129** (3), 797 (1972).
88. L. M. Lanier, M. A. Gates, W. Witke et al., *Neuron* **22** (2), 313 (1999).
89. C. G. Dotti, C. A. Sullivan, and G. A. Banker, *J Neurosci* **8** (4), 1454 (1988).
90. B. Gigant, C. Wang, R. B. Ravelli et al., *Nature* **435** (7041), 519 (2005).
91. L. Dehmelt, F. M. Smart, R. S. Ozer et al., *J Neurosci* **23** (29), 9479 (2003).
92. K. J. De Vos, A. J. Grierson, S. Ackerley et al., *Annu Rev Neurosci* **31**, 151 (2008).
93. K. E. Zinsmaier, M. Babic, and G. J. Russo, *Results Probl Cell Differ* **48**, 107 (2009).
94. A. S. Geiger, A. C. Rice, and S. M. Shapiro, *Neonatology* **92** (4), 219 (2007).
95. J. W. Conlee and S. M. Shapiro, *Acta Neuropathol* **93** (5), 450 (1997).
96. K. Gkoltsiou, M. Tzoufi, S. Counsell et al., *Early Hum Dev* **84** (12), 829 (2008).
97. P. Nopoulos, I. Torres, M. Flaum et al., *Am J Psychiatry* **152** (12), 1721 (1995).
98. M. E. Shenton, R. Kikinis, F. A. Jolesz et al., *N Engl J Med* **327** (9), 604 (1992).
99. T. Stepanova, J. Slemmer, C. C. Hoogenraad et al., *J Neurosci* **23** (7), 2655 (2003).
100. B. Gigant, C. Wang, R. Ravelli et al., *Med Sci (Paris)* **21** (10), 814 (2005).
101. F. E. Poulain and A. Sobel, *Mol Cell Neurosci* **34** (2), 137 (2007).
102. B. Zhang, A. Maiti, S. Shively et al., *Proc Natl Acad Sci U S A* **102** (1), 227 (2005).
103. O. Mercado-Gomez, P. Ferrera, and C. Arias, *J Neurosci Res* **78** (4), 553 (2004).
104. K. M. Kollins, R. L. Bell, M. Butts et al., *Neural Dev* **4**, 26 (2009).
105. M. DiTella, F. Feiguin, G. Morfini et al., *Cell Motil Cytoskeleton* **29** (2), 117 (1994).
106. H. Felgner, R. Frank, J. Biernat et al., *J Cell Biol* **138** (5), 1067 (1997).
107. T. L. Briones, J. Woods, M. Wadowska et al., *Behav Brain Res* **168** (2), 261 (2006).
108. A. Reyna-Neyra, I. Camacho-Arroyo, P. Ferrera et al., *Brain Res Bull* **58** (6), 607 (2002).
109. N. S. Jalava, F. R. Lopez-Picon, T. K. Kukko-Lukjanov et al., *Int J Dev Neurosci* **25** (2), 121 (2007).
110. S. L. Gonzalez, J. J. Lopez-Costa, F. Labombarda et al., *Cell Mol Neurobiol* **29** (1), 27 (2009).
111. O. Hansson, H. Zetterberg, P. Buchhave et al., *Lancet Neurol* **5** (3), 228 (2006).
112. H. Hampel, A. Goernitz, and K. Buerger, *Brain Res Bull* **61** (3), 243 (2003).
113. D. Terwel, R. Lasrado, J. Snauwaert et al., *J Biol Chem* **280** (5), 3963 (2005).
114. M. Vershinin, B. C. Carter, D. S. Razafsky et al., *Proc Natl Acad Sci U S A* **104** (1), 87 (2007).
115. C. Y. Chung, J. B. Koprich, H. Siddiqi et al., *J Neurosci* **29** (11), 3365 (2009).
116. M. Vershinin, J. Xu, D. S. Razafsky et al., *Traffic* **9** (6), 882 (2008).
117. I. Cuchillo-Ibanez, A. Seereeram, H. L. Byers et al., *FASEB J* **22** (9), 3186 (2008).
118. W. Wang, D. Takezawa, S. B. Narasimhulu et al., *Plant Mol Biol* **31** (1), 87 (1996).
119. R. Mallik, B. C. Carter, S. A. Lex et al., *Nature* **427** (6975), 649 (2004).
120. S. Hanz and M. Fainzilber, *J Neurochem* **99** (1), 13 (2006).
121. F. Ferreirinha, A. Quattrini, M. Pirozzi et al., *J Clin Invest* **113** (2), 231 (2004).
122. A. D. Pilling, D. Horiuchi, C. M. Lively et al., *Mol Biol Cell* **17** (4), 2057 (2006).