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Epigenetic changes in a mouse model of Rett Syndrome: optimizing a technique to evaluate DNA methylation

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Resumo

A Síndrome de Rett é uma doença genética causada, em 90% dos casos, por uma mutação no gene *MECP2*, localizado no cromossoma X, que codifica a proteína com o mesmo nome. A Mecp2 tem inúmeras funções, em particular ser um importante "*reader*" da metilação de ADN. Sabe-se que a metilação de ADN está alterada na epilepsia; a hipótese da metilação na epileptogénese sugere que convulsões em si podem induzir modificações epigenéticas na cromatina agravando a condição epileptogénica. A adenosina, anticonvulsionante endógeno, foi implicada na epileptogénese pela sua capacidade de modular a metilação do ADN no cérebro. Em condições epiléticas os níveis de adenosina estão reduzidos, impedindo a sua ação anticonvulsionante a partir do controlo do tónus inibitório sináptico e pela promoção da hipometilação do ADN no processo epileptogénico. Recentemente, foi demonstrado que a adenosina está diminuída no hipocampo e córtex de modelos de ratinho Mecp2 knockout, hipotetizando-se, assim, que a condição epilética na RTT pode ter contribuição da subregulação de adenosina. Com a o objetivo de explorar uma possível relação entre a severidade da condição epilética associada a cada fenótipo RTT e mudanças na metilação do ADN no Sistema Nervoso Central e periferia (sangue), decidimos otimizar a metodologia necessária para observar mudanças na metilação do ADN em dois fenótipos diferentes (mild – symptomatic heterozygous (HET) females and severe – knockout (KO) symptomatic males) de ratinho modelo Mecp2 Knockout (B6.129P2 (C)-Mecp2tm1.1Bird/J). Assim, diferentes métodos de purificação de ADN foram testados, usando dois "*buffers"* de lise, procedidos de dot blot usando o anticorpo N6-metiladenosina. No fim, podemos confirmar que deteção foi possível com o uso de dot blot para as diferentes amostras e concentrações. Comparando os buffers, chegámos à conclusão inicial que o TDB, face ao RIPA, oferece resultados melhores no que toca à concentração e ratios da espectrofotometria obtidos, indicativos de maior pureza.

Palavras-chave: Síndrome de Rett (RTT); *MECP2*; Metilação de ADN; Adenosina

Abstract

RTT is a genetic disorder caused, in 90% of cases, by a genetic mutation in the *MECP2* gene, located on the X chromosome, which encodes the protein of the same name. *MECP2* has innumerous roles, namely being an important reader of DNA methylation. It is known that DNA methylation is altered in epilepsy; the methylation hypothesis of epileptogenesis suggests that seizures by themselves can induce epigenetic chromatin modifications and thereby aggravate the epileptogenic condition. Adenosine, an endogenous anticonvulsant, has been implicated in epileptogenesis by its ability to modulate DNA methylation in the brain. In epileptic conditions adenosine levels are decreased, which impairs its proper anticonvulsant action by controlling both synaptic inhibitory tonus and epileptogenesis process by promoting DNA hypomethylation. Recently, it was shown that adenosine is decreased in the hippocampus and cortex of Mecp2 knockout mouse model hypothesizing that epileptic condition in RTT could have a contribution of adenosine deregulation. With the intention of exploring a possible relationship between epileptic condition severity associated with each RTT phenotype and DNA methylation changes in central nervous system and at periphery (blood), we set ourselves to optimize the methodology needed to observe changes on DNA methylation in two different phenotypes (mild – symptomatic heterozygous (HET) females and severe – knockout (KO) symptomatic males) of the Mecp2 KO mouse model (B6.129P2 (C)-Mecp2tm1.1Bird/J). With this in mind, different DNA purifications methods were tested, using two different lyses buffers, proceeded by a dot blot technique using the the N6-methyladenosine antibody (6ma). In the end, we can confirm that detection was possible by the use of a dot blot technique for the different samples and concentrations. When comparing the buffers we've reached an initial conclusion that TDB, opposed to RIPA, offers better results when it comes to the achieved DNA concentration and the microvolume spectrophotometry ratios, indicators of purity.

Keywords: Rett Syndrome (RTT); *MECP2*; DNA methylation; Adenosine

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Índice

Introduction

1. Rett Syndrome

Rett Syndrome (RTT) is a genetic disorder caused, in 90% of cases, by a genetic mutation in the *MECP2* gene, located on the X chromosome. It almost always occurs as a new mutation and affects almost exclusively girls; boys who have a similar mutation typically die shortly after birth. The *MECP2* is a gene that encodes a protein with the same name, an important reader of DNA methylation. This protein is involved in the regulation of gene activity (expression) by modifying chromatin, the complex of DNA and protein that packages DNA into chromosomes. The MeCP2 protein is present in cells throughout the body, although it is particularly abundant in brain cells [3].

Mice lacking *Mecp2* or carrying RTT-associated mutations in *Mecp2* have been developed, and the majority of clinical features present in human RTT patients are observed in these *Mecp2*-mutant mice. Loss of *Mecp2* from only the Central Nervous System (CNS) either embryonically or postnatally results in a diverse array of phenotypes, emphasizing its importance in neuronal function and maintenance. *Mecp2*-mutant mice also display pathological and histological features found in RTT patients, such as a smaller brain size and a reduction in the number and length of dendrites [9;10;11;12;13].

RTT manifestations exceed the CNS and it is possible to observe an extensive array of phenotypes present in people with the condition [6]. This is due to the mutation in the gene not only affecting the brain but also the periphery. Regarding RTT symptoms progression, it is possible to establish four different stages. Stage I, called early-onset, typically begins between 6 and 18 months of age. This stage is often overlooked because symptoms of the disorder may be somewhat vague. Stage II, the rapid destructive stage, usually begins between ages 1 and 4 and may last for weeks or months. Its onset may be rapid or gradual as the child loses purposeful hand skills and spoken language. Some girls also display autistic-like symptoms such as loss of social interaction and communication. Walking may be unsteady and initiating motor movements can be difficult. Slowed head growth is usually noticed during this stage. Stage III, the plateau

or pseudo-stationary stage, usually begins between ages 2 and 10 and can last for years. Apraxia, motor problems, and seizures are prominent during this stage. Many girls remain in this stage for most of their lives. Stage IV, or the late motor deterioration stage, can last for years or decades. Prominent features include reduced mobility, curvature of the spine, and muscle weakness, rigidity, spasticity, and increased muscle tone with abnormal posturing of arms and legs [8]. Currently there is no known cure for RTT and the available treatments are limited and directed towards improving function and addressing symptoms [1;2].

Nearly 70-90 percent of RTT patients experience seizures. In most cases, seizures appear during stages 2 or 3 of the disease, which occur when the patient is around age 4. The maximal seizure frequency is seen between ages 7 and 12 [7]. Recently, our lab showed that the adenosinergic modulation of synaptic transmission is compromised in *Mecp2* knockout mouse, a widely used mouse model of RTT. Adenosine appears to be decreased in the hippocampus and cortex, accompanied by an increased A_1R and decreased $A_{2A}R$ expressions. This leads us to hypothesize that epileptic condition in RTT could have a contribution of adenosine deregulation [5]. Indeed, adenosine deficiency has been identified as a pathologic hallmark of the epileptic brain [33]. Seizures have been associated with a rise in adenosine, which acts as a seizure terminator. When endogenous seizure control mechanisms fail, *status epilepticus* results, which can cause major injury to the brain and which can trigger subsequent epileptogenesis. A clinical example highlighting the importance of adenosine-related control mechanisms is the ophylline-induced *status epilepticus* in susceptible individuals, which can develop into a life-threatening condition. Because the ophylline is an adenosine receptor antagonist, those findings show that seizures are normally kept under control by endogenous adenosine acting on adenosine receptors. This example, as well as experimental data in rodents, shows a critical role of adenosine A_1R in preventing seizure spread [14;15;16;17;18;19;20].

2. Adenosine

Adenosine is an organic compound that occurs widely in nature in the form of diverse derivatives. The molecule consists of an adenine attached to a ribose via a β-N9-glycosidic bond. Adenosine is one of four nucleoside building blocks to DNA and RNA, which are essential for all life. Its derivatives include the energy carriers adenosine mono-, di-, and triphosphate, also known as AMP/ADP/ATP. It modulates many physiological processes and its cellular signaling occurs through four known adenosine receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3 (Fig. 1). All adenosine receptor subtypes are G-protein-coupled receptors. The four receptor subtypes are further classified based on their ability to either stimulate or inhibit adenylate cyclase activity. The A_1 receptors couple to $G_{i/o}$ and decreases cAMP levels, while the A_2 adenosine receptors couple to G_s, which stimulates adenylate cyclase activity. In addition, A_1 receptors couple to G_o, which has been reported to mediate adenosine inhibition of Ca^{2+} conductance, whereas A_{2B} and A_3 receptors also couple to G_q and stimulate phospholipase activity. Extracellular adenosine concentrations from normal cells are approximately 300 nM; however, in response to cellular damage (e.g., in inflammatory or ischemic tissue), these concentrations are quickly elevated (600–1,200 nM). Thus, in regard to stress or injury, the function of adenosine is primarily that of cytoprotection preventing tissue damage during instances of hypoxia, ischemia, and seizure activity.

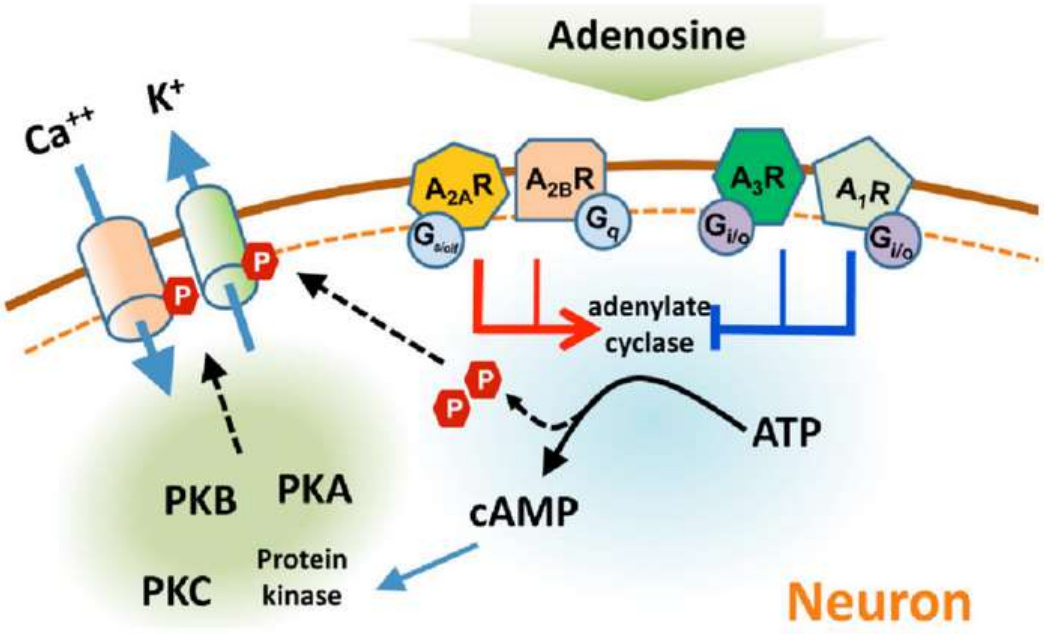

Fig 1. – Adenosine exerts its effects through its four subtypes of receptors, A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. Adenosine activation of $G_{i/o}$-coupled A_1Rs and A_3Rs inhibits activity of cAMP cyclase. In contrast, adenosine-activation of $G_{s/olf}$-coupled $A_{2A}R$ and G_q-coupled $A_{2B}R$ enhances the activity of cAMP cyclase. Therefore, four subtypes of adenosine receptors manipulate cellular cAMP levels, and thus consequently affect downstream protein kinase pathways, i.e. PKA, PKC and PKB [45].

Adenosine is an endogenous anticonvulsant and seizure terminator of the brain. Adenosine affects seizure generation, as well as the development of epilepsy and its progression. Maladaptive changes in adenosine metabolism play a major role in epileptogenesis. Neuronal excitability in the brain is modulated by the activation of G-protein coupled adenosine receptors (namely A_1 and A_{2A}). Neuronal excitability therefore depends on the equilibrium of receptor effects, expression levels in different brain regions, and their availability for receptor activation. In general, the epileptic state is marked by decreased A_1R signaling and/or enhanced $A_{2A}R$ signaling. It is currently unknown whether modifications in adenosine receptor expression are cause or consequence of epilepsy [32].

2.1. Adenosine A_1R

A_1 receptors are mainly present in the cortex, hippocampus, cerebellum, nerve terminals, spinal cord, and glia [35]. The multitude of physiological effects caused by it include inhibition of neurotransmitter release, reduction of neuronal excitability, sedation, anticonvulsant and anxiolytic effects, analgesia and regulation of sleep [34].
These effects are mediated through A_1 receptors coupling to G_i/G_o proteins, inhibition of AC, activation of phospholipase-C (PLC)β and, particularly in neurons, activation of potassium channels and deactivation of Q-, P-, and N-type Ca^{2+} channels.
Research has shown that a reduction in A_1R density and a deficiency of endogenous adenosine based seizure control mechanisms occur in the rat kindling model of epilepsy, indicating a failure of endogenous seizure control mechanisms. Receptor knockout designs have demonstrated that mice without A_1R have spontaneous electrographic seizures and develop fatal *status epilepticus* after intrahippocampal injection of kainic acid or after a traumatic brain injury. These experiments exemplify that A_1R activation is necessary for the suppression of seizures and their spread and suggest that disruption of A_1R signaling is dynamically linked to the pathophysiology of epilepsy [21;22;23;32;33].

2.2. Adenosine $A_{2A}R$

The A_{2A} receptor is highly expressed in the striatum, mainly present in GABAergic striatopallidal neurons, corticostriatal glutamatergic terminals, and cholinergic interneurons, but it is also detectable in the olfactory tubercle, cerebral cortex, hippocampus, neurons, and glial cells, where it induces excitotoxicity by affecting release of glutamate, activation of glia and infiltration of immune cells from the periphery, through blood brain barrier passage [34;37;38].

The A_{2A} receptor generally couples to G_s proteins to increase cAMP levels, but in the brain it stimulates G_{olf}, a specific G_s protein in neurons. The signaling cascade starting from cAMP and PKA regulates different proteins such as cAMP responsive element binding protein (CREB) and DA- and cAMP-regulated phosphoprotein (DARPP-32) [42]. In addition, the A_{2A} receptor, with its long C-terminus, could also bind to different

accessory proteins like D_2 receptors, ADP-ribosylation factor nucleotide site opener (ARNO), α-actinin, translin-associated protein X (TRAX) and ubiquitin-specific protease (USP4). A_{2A} receptor activation may also trigger the Ras/Raf-1/MEK/ERK pathway through PKA-dependent or independent mechanisms [34;43].

A reduction in the synaptic expression of A_{2A} receptors can mitigate synaptotoxic consequences of the synaptic pool of adenosine [32;39;40;41]. This function is directly linked to the neuronal release of adenosine or its precursor ATP, which leads to neuronal hyperexcitability, which is likely dependent on increased synaptic A_{2A} receptor activation. This aggravation of synaptotoxicity could contribute to the exacerbation of epilepsy. Genetic changes in the A_{2A}R gene have been linked with acute encephalopathy and seizures in children, indicating that dysregulation of A_{2A}Rs may play a role in seizure progression and excitotoxic brain injury. In line with a possible pro-convulsive role of A_{2A}Rs, A_{2A}R knockout mice were partially resistant to limbic seizures, further suggesting that promotion of the epileptic state is a consequence of increased A_{2A}R activation [32].

2.3. Adenosine A_{2B}R and A_3R

A_{2B} receptors are present in astrocytes, neurons, and microglia, but their role in the Central Nervous System is less well characterized in comparison to the other receptors. For A_3 receptors, a low level of expression has been detected in the brain, in which it was detected in the cortex, thalamus and hypothalamus, hippocampus, motor nerve terminals, retinal ganglion cells, pial and intracerebral arteries and glia. Based on their low expression levels in the brain and lower affinities for adenosine, A_{2B}Rs and A_3Rs do not appear to play a direct role in epilepsy. However tissue from human resected epileptic tissue transplanted into *Xenopus* oocytes showed that selective A_{2B}R and A_3R antagonists decreased the run-down of GABA currents. Thus, it has been suggested that cortical adenosine A_{2B} and A_3 receptors alter the stability of GABAA receptors and thus potentially calibrate neuronal excitability [44].

3. DNA Methylation

In addition to adenosine receptor dependent effects, adenosine itself acts as a biochemical metabolite and plays a major role in the transmethylation pathway. Because adenosine is a product of transmethylation it assumes a unique role as a regulator of DNA methylation [36]. The extent of the DNA methylation of genomic DNA as well as the methylation pattern of many gene-regulatory areas are important aspects with regard to the state of genetic information, especially their expression. DNA methylation impacts the function of DNA by activating or repressing the transcriptional activity of a gene. There is growing evidence that aberrant methylation is associated with many serious pathological consequences. DNA methylation relies on the donation of a methyl group from S-adenosylmethionine. The product of methyl group transfer to DNA is S-adenosylhomocysteine, which is then cleaved into adenosine and homocysteine by SAH hydrolase. The equilibrium of this reaction is determined by the local adenosine concentration in the microenvironment of the cell nucleus (Fig.2) [36].

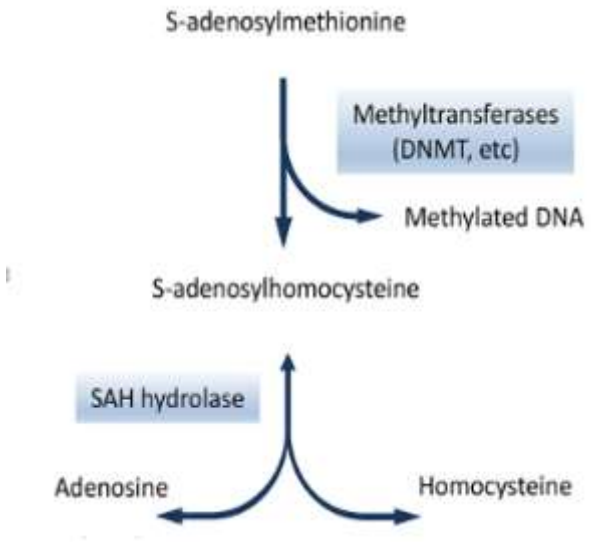

Fig 2. – Transmethylation pathway. Because adenosine is one of the products of the process the equilibrium of this reaction is determined by the adenosine concentration.

The methylation hypothesis of epileptogenesis suggests that seizures by themselves can induce epigenetic chromatin modifications (e.g. DNA hypermethylation) and thereby aggravate the epileptogenic condition. In epileptic conditions, adenosine levels are decreased which impairs its proper anticonvulsant action by controlling both synaptic inhibitory tonus and epileptogenesis process by promoting DNA hypomethylation. Despite the knowledge about DNA methylation in RTT being scarce, the decreased adenosine present in the disease might justify the DNA methylation modifications in patients' brains.

It was widely accepted for a long time that 5-methylcytosine was the only form of DNA methylation in mammalian genomes. As a methylated form of the DNA base cytosine, it regulates gene transcription and takes several other biological roles. When cytosine is methylated, the DNA maintains the same sequence, but the expression of methylated genes can be altered. 5-Methylcytosine is incorporated in the nucleoside 5-methylcytidine. Due to its characteristics this target has been studied and used extensively in the study and marking of DNA methylation. However recently N6-methyladenosine (m6A) has been identified as another form of DNA methylation[29]. *N6*-Methyladenosine was originally identified and partially characterized in the 1970s, and it is an abundant modification in mRNA and DNA [28]. It is found within some viruses and most eukaryotes including mammals, insects, plants and yeast. The methylation of adenosine is directed by a large m6A methyltransferase complex containing METTL3 as the SAM-binding sub-unit. The biological functions of m6A are mediated through a group of RNA binding proteins that specifically recognize the methylated adenosine on RNA [46]. Setting this DNA methylation as a target can help us retrieve a wider array of information from our scope of interest, due to its connection to adenosine. For this we decided to detect DNA methylation using the Anti-N6-methyladenosine Antibody (6ma), which can become pivotal in the study of DNA methylation and adenosine changes.

4. Detecting and quantifying DNA methylation

When it comes to the detection of DNA methylation several techniques have been used in the past, namely the southern blot and the dot blot.

4.1. Southern blot

The method is named after the British biologist Edwin Southern, who first published it in 1975 [31]. It was the first method that was used in the detection of a specific DNA sequence in various DNA samples. The method involves the transfer of DNA fragments from an electrophoresis gel to a membrane causing immobilization of the DNA fragments and bands are produced. After immobilization, the DNA fragments could be subjected to hybridization analysis, enabling the bands with sequence similarity to a labeled probe. Oligonucleotides, similar to the target sequence are designed. These oligonucleotides are chemically synthesized, radiolabeled, and then used for the cloned DNA fragments. Sequences hybridizing with the hybridization probe are further analyzed to obtain the full-length sequence of the gene of interest [24]. Southern blotting is a suitable technique to analyze long stretches of DNA. The expression levels and expression profiles are better detected as compared to the other genomic analyses methods. The biggest drawback of this method is that it requires a large quantity of high-quality DNA.

4.2. Dot blot

A dot blot is a simple way to test for the presence of a protein of interest in a sample, namely DNA. It relies on the same principle that many other immunological techniques: the recognition and binding of an antigen by an antibody. Briefly, dot blot utilizes a dry nitrocellulose or PVDF membrane that has been "dotted" with sample homogenate. The membrane is then blocked for non-specific binding using a blocking buffer, followed by incubation with a primary antibody specific to the protein of interest. This step is then followed by incubation with a secondary antibody that allows visual detection and quantification of the target protein through methods such as chemiluminescence or fluorescence. Advantageously, the Dot blot method does not require the separation of

bands on the solid support medium (agarose), electrophoresis is not required and it does not involve immobilization of the biomolecules from a gel matrix to the filter membrane. However, dot blot method does not give any qualitative information about the target biomolecules' size and molecular weight, and it does not provide a basis for comparing an original and a modified target biomolecule within the same slot. All things considered the dot blot, while not as informative as other techniques, is an inexpensive, quick and useful technique widely used for assessing a large number of samples where the simple detection of DNA is the goal, therefore we opted for this option.

Aim

In the future we aim to study DNA methylation, by detecting and comparing it in the Central Nervous System and at periphery (blood) using samples of two different phenotypes [mild – symptomatic heterozygous (HET) females and severe – knockout (KO) symptomatic males] of the Mecp2 KO mouse model [B6.129P2 (C)-Mecp2tm1.1Bird/J]. With this knowledge, we can raise the question whether the effects on the methylation and adenosine levels present in the blood are in any way correlated to those happening in the brain. If correlation is found it allows for indirect evaluation through the peripheral levels, making adenosine augmentation strategies easier to execute and manage with the ability to control adenosine levels and consequent avoidance of excessive increases in adenosine.

With this in mind we set ourselves to extract the DNA from these samples and optimize a technique to detect and quantify DNA methylation, which has not been implemented before. This technique would optimize the study of epigenetic changes on DNA methylation occurring in different RTT phenotypes and would consist as a stepping stone to achieve a possible relationship between epileptic condition severity and DNA methylation changes in the central nervous system and at periphery (blood). Achieving this clarification could support the viability of adenosine augmentation therapies and how it should be explored in different cases of RTT.

Ethical implications

This project involves laboratory animals (mice models) and thereby has to follow the current legislation. The mice were handled according to European Community guidelines and Portuguese law on Animal Care.

Methodology

1. Animal samples

Experiments were performed in samples collected in previous experiments from the mouse model of RTT (B6.129P2)C)-Mecp2tm1.1Bird/J [MECP2 knockout; Mecp2−/y], JAX #003890; (Guy et al., 2001) during the symptomatic stage (males be-tween 6 and 10 weeks old; females between 26 and 28 weeks); wild type (WT) littermates were used as control. The genotype of animals was determined by PCR analysis, as previously described (Guy et al., 2007). The animals were housed on a 12 h light/dark cycle, with food and water provided ad libitum. Throughout the experimental work, care was taken to minimize the number of animals sacrificed. All animals were handled according to the Portuguese law on Animal Care and European Community guidelines (86/609/EEC). Mice were sacrificed by decapitation under deep isoflurane anesthesia. After decapitation, the mice brains were quickly removed, hemi-sected and both hippocampi and cortexes were dissected free within ice-cold artificial cerebrospinal fluid (aCSF) solution: NaCl 124 mM; KCl 3 mM; NaH2PO4 1.25 mM; NaHCO3 26 mM; MgSO4 1 mM; CaCl2 2 mM; and glucose 10 mM, previously gassed with 95% O2 and 5% CO2, pH 7.4.

In the first group a total of 2 samples of cortex, 2 of hippocampus and 2 of blood were collected from healthy mice. In the second group a total of 19 samples of cortex and 19 of hippocampus were collected. The snap-frozen cortex and hippocampus samples from the first group mice were first disrupted with a Teflon pestle in Radio-Immunoprecipitation Assay (RIPA) buffer containing: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethyl-enediamine tetra-acetic acid (EDTA), 0.1% SDS and 1%Triton X-100 and protease inhibitors cocktail (Mini-Complete EDTA-free; Roche Applied Science, Penzberg, Germany). In the second group the buffer used was instead tail digestion buffer (TDB), containing: 50 mM KCl; 10 mM Tris-HCl (pH=9.0); 0.1 % Triton X-100; Proteinase K with a final concentration of 0.15 mg/ml. We kept the buffered samples overnight at 55ºC and then raised the temperature to 96ºC for half an hour. All lysates were then cooled, vortexed and sonicated (3 cycles of 15 s), and clarified by centrifugation (13,000 g, 10 min).

2. DNA isolation and purification by Phenol-Chloroform-Isoamyl - alcohol 25:24:1

An equal volume of ice-cold Phenol-Chloroform-Isoamyl (PCI) 25:24:1 was added to each sample, followed by centrifugation at 15000 x g for 5 minutes to separate it in phases. The aqueous layer was transferred to a fresh microcentrifuge tube to which was again added an equal volume of chloroform-isoamyl alcohol 24:1, followed by centrifugation at 15000 x g for 5 minutes. The subsequent aqueous layer was transferred to a fresh microcentrifuge tube, adding to each sample 50µL 3M Na-acetate and 1mL 96% ethanol (at -20°C) and mixing thoroughly. The samples were incubated at -80°C, during at least 2h, thawed and centrifuged for 30 minutes. The supernatant was removed by pipetting, then the samples were washed with 70% ethanol. The DNA samples were then dissolved in 30uL of H2O.

3. Microvolume spectrophotometry

We then proceeded to quantify the DNA present in the resulting samples and observed their purity ratios 260/280 and 230/260 with a Spectrophotometer Nanodrop, Thermo Scientific; Model:ND-2000. With this we intend to get information on possible sample contamination, possibly coming from the presence of RNA or protein.

Microvolume spectrophotometers (MVS) are used for the analysis of nucleic acid (NA) samples. They require a small sample volume (0.5–2.0 µl) and are economical, convenient and widely commercially available. Typically, they can measure NA concentrations as low as 1 ng/µl and they are compatible with various assays for all types of NA and proteins. Nucleic acid concentrations are determined by measuring the absorbance of ultraviolet light. Derived from the Beer-Lambert law, the amount of light absorbed at 260 nm is proportional to the concentration of nucleic acid in solution. Additionally, as an indicator of sample purity, the ratios of the absorbance values of 260 nm vs 280 nm (A260/A280) and the 260 nm vs 230 nm (A260/A230) can be determined.

The A260/A280 provides insight regarding the type of nucleic acid present (dsDNA or RNA) as well as providing a rough indication of purity. Typically, protein contamination

can be detected by a reduction of this ratio; RNA contamination can be detected by an increase of this ratio. In buffered solutions, pure DNA has an A260/A280 of 1.85–1.88 and pure RNA has a ratio of around 2.1.

The A260/A230 is a sensitive indicator of contaminants that absorb at 230 nm. These contaminants are significantly more numerous than those absorbing at 280 nm, and include chaotropic salts such as guanidine thiocyanate (GTC) and guanidine hydrochloride (GuHCl), EDTA, non-ionic detergents, proteins and phenol. Substances like polysaccharides or free floating solid particles like silica fibers absorb at this wavelength, but will have a weaker effect. In buffered solutions, pure DNA has slightly higher A260/A230 ratios than RNA, with a value of 2.3–2.4 commonly reported for DNA and 2.1–2.3 for RNA. A260/A230 ratios typically produce a higher standard deviation than A260/A280 ratios and should be interpreted with care [25;26].

4. Dot Blot (Following the protocol from Wu et al., 2016)

First, DNA samples were denatured at 95 degrees for 5 min, cooled down on ice, neutralized with 10% volume of 6.6 M ammonium acetate. Samples were spotted on the membrane (Amersham Hybond-N+, GE) and air dry for 5 min, then UV-crosslink (2× auto-crosslink, 1800 UV Stratalinker, STRATAGENE). Membranes were blocked in blocking buffer (5% milk, 1% BSA, PBST) for 2 h at room temperature, incubated with 6mA antibodies (202-003, Synaptic Systems, 1:1000) overnight at 4 degrees. After 5 washes, membranes were incubated with HRP linked secondary anti-rabbit IgG antibody (1:5,000, Cell Signaling 7074S) for 30 min at room temperature. Signals were detected with ECL Plus Western Blotting Reagent Pack (GE Healthcare).

Results

1. Spectrophotometry

In order to optimize the protocol of DNA extraction, we tested two different lyses buffers, the RIPA buffer and the TDB lyses buffer.

Spectrophotometry for the samples treated with RIPA buffer (Table 1), revealed highest concentration of DNA in the cortex (with an average of 322.35 ng/µl), followed by the hippocampus (average of 209.45 ng/µl) and with lowest being the blood (average of 107.25 ng/µl). The A260/A280 provides insight regarding the type of nucleic acid present, in this case DNA, as well as providing a rough indication of purity. Typically, protein contamination can be detected by a reduction of this ratio; as such, looking at the Abs260/280 ratios it is expected for the contamination to have happened by remaining protein content. The contamination appears to be lowest in the hippocampus, with values close to the ideal, however when it comes to the cortexes it is more apparent. The same goes for the blood with very high ratios that makes us unsure about what components caused the contamination.

Table 1 – DNA concentration, Abs260/280, Abs260/230 ratios of different samples tested using RIPA lyses buffer. Ca and Cb are cortex samples, Ha and Hb hippocampus samples, Ba and Bb blood samples.

Samples (RIPA)	DNA concentration ng/µl	Abs260/280	Abs260/230
Ca	306.2	1.39	0.55
Cb	338.5	1.41	0.51
Ha	224.8	1.64	0.68
Hb	194.1	1.63	0.61
Ba	167.5	3.29	2.19
Bb	47.0	3.60	3.00

Using TDB buffer (Table 2), higher concentration of DNA was obtained in the cortexes (with an average of 1018.58 ng/µl) when compared to the hippocampus (average of 327.63 ng/µl). With this buffer we can notice a clearly higher concentration of DNA when compared to the RIPA buffer in both cortexes (1018.58 ng/µl vs 322.35 ng/µl) and hippocampus (327.63 ng/µl vs 209.45 ng/µl). We can spot some abnormally low concentrations in some samples, namely C8 and H6, which might have resulted from human error during the process. Importantly, the Abs260/280 ratios show relatively similar levels of purity between the two types of sample, with most of the samples presenting ratios close to the values accepted as "pure" mentioned before (1.85–1.88). Once again the contamination present is expected to be caused by remaining protein content. The TDB buffer offers better results, with an average Abs260/280 of 1,70, opposed to the RIPA, with an average Abs260/280 of 1,52.

Table 2 – DNA concentration, Abs260/280, Abs260/230 ratios of different samples tested using TDB lyses buffer. C1-C19 are cortex samples: C1-C5 are female wt; C6-C9 female het; C10-C14 male wt; C15-C19 male ko. H1-H19 are hippocampus samples: H1-H5 are female wt; H6-H9 female het; H10-H14 male wt; H15-H19 male ko.

Samples (TDB)	DNA Concentration ng/µl	Abs260/280	Abs260/230
C1	865.5	1.76	1.70
C2	851.9	1.80	1.89
C3	1330.9	1.68	1.64
C4	1357.5	1.32	1.38
C5	634.1	1.53	1.37
C6	555.8	1.50	1.41
C7	1704.4	1.52	1.33
C8	87.8	1.63	1.69
C9	641.3	1.79	1.92
C10	904.3	1.72	1.47
C11	2845.6	1.65	1.37
C12	1755.6	1.61	1.35

C13	527.9	1.80	1.40
C14	700.8	1.96	1.88
C15	995.1	1.68	1.44
C16	1356.3	1.79	1.83
C17	1199.0	1.70	1.69
C18	360.7	1.77	1.59
C19	678.5	1.71	1.50
H1	446.9	1.79	1.75
H2	122.5	1.54	1.40
H3	446.5	1.80	1.72
H4	283.7	1.82	1.67
H5	236.2	1.41	1.35
H6	19.3	1.68	1.02
H7	259.7	1.73	1.67
H8	504.2	1.61	1.11
H9	459.7	1.79	1.87
H10	545.8	1.68	1.57
H11	263.0	1.57	1.61
H12	541.2	1.81	1.85
H13	168.8	1.60	1.48
H14	171.7	1.74	1.57
H15	214.1	1.75	1.73
H16	478.2	1.82	1.77
H17	307.4	1.71	1.56
H18	320.7	1.76	1.68
H19	435.8	1.76	1.65

2. Dot Blot

From the spectrophotometry, we proceeded to use the dot blot technique to evaluate the presence of DNA methylation in the samples treated with RIPA buffer, coupled with a sample treated with TDB buffer for comparison (Fig.3).

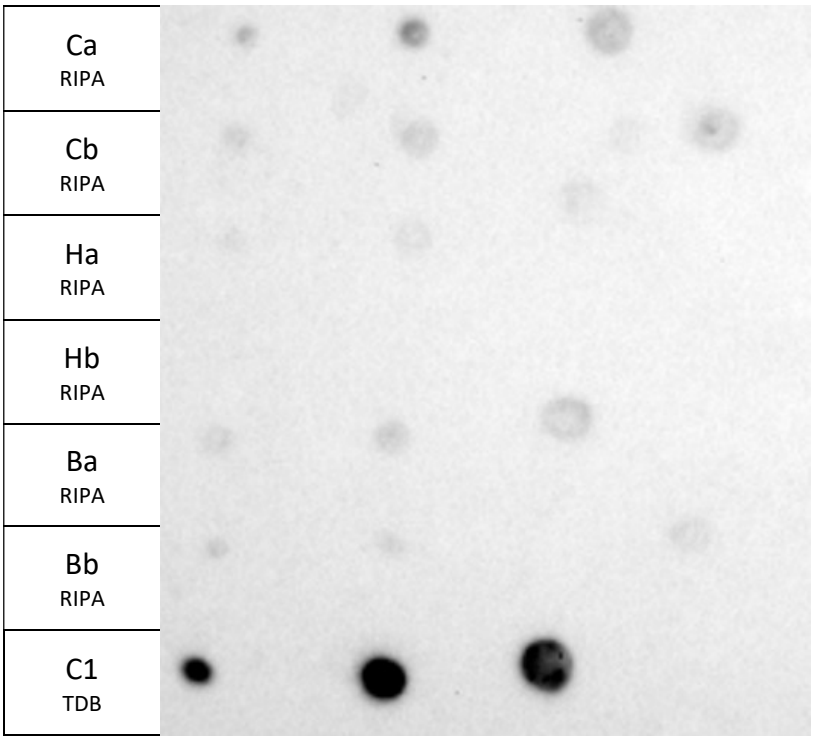

Fig 3. – Detection of m6A on DNA samples in Dot blot – A darker shade means a higher density of antibody m6a on DNA. The samples Ca and Cb are cortex, Ha and Hb hippocampus and Ba and Bb samples of blood, corresponding to the samples treated with RIPA. C1 is a cortex sample tested with TDB for comparison. There's three applications of the same sample in each line, with increasing volumes of sample spotted.

We can see that the DNA purified with TDB buffer, C1, presents a significantly darker shade in the spotting when compared to the samples treated with RIPA buffer, which is indicative of a higher presence of m6a. This means more DNA methylation was detected.

Discussion

Our data shows that the levels of DNA concentration and purity achieved with the TDB buffer were higher than the ones obtained with the RIPA buffer, suggesting that the TDB buffer would be a better choice to use in future work. In addition, the quantification obtained in the dot blot shows a higher presence of antibody signaling for the sample tested with TDB, further reinforcing its use.

The RIPA buffer is composed by: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethyl-enediamine tetra-acetic acid (EDTA), 0.1% SDS and 1%Triton X-100 and protease inhibitors cocktail. TDB, on the other hand, contains: 50 mM KCl; 10 mM Tris-HCl (pH=9.0); 0.1 % Triton X-100; Proteinase K with a final concentration of 0.15 mg/ml. We can speculate the role of proteinase K present in the TBD, and not in RIPA buffer, as a factor in these results. Proteinase K is a protein highly resistant to denaturation by heat, chaotropic salts, and detergents and it will continue to function in their presence provided the temperature/concentration do not cross a fairly high threshold. Given its relative resistance to high temperatures, it is often used at higher temperatures (greater than 50°C), because many of the nucleases that would chew up DNA would be inactivated. In addition to its stability, Proteinase K is also highly-touted for its broad specificity. Proteins will be completely digested if the incubation time is long and the protease concentration high enough. The benefit of using proteinase K during DNA extraction is its ability to degrade a wide range of damaging nucleases. It is also great for digesting surface proteins on the cell membrane. Addition of Proteinase K to nucleic acid preparations rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification. Proteinase K is used for the destruction of proteins in cell lysates (tissue, cell culture cells) and for the release of nucleic acids, since it very effectively inactivates DNases and RNases. The residual activity is sufficient to digest proteins and can possibly contaminate nucleic acid preparations. Therefore, the digestion with Proteinase K for the purification of nucleic acids is usually performed in the presence of EDTA (inhibition of metal-ion dependent enzymes such as nucleases), which was not the case and might constitute one of the causes for the contamination

still present in some of the samples [27]. What we performed instead was the inactivation of Proteinase K by the use of high temperature (96ºC).

The number of total samples is reduced and blood samples were never treated with the TDB buffer, prompting further studying to solidify the use of TDB. It is also important to consider that we carried out our spectrophotometry in water. In spectrophotometric measurements carried out in water A260/A280 ratios tend to be 0.3–0.4 units lower than in buffered and mildly alkaline solutions. In our case we used RNA-free water to decrease this impact, but measuring them in Tris buffer could be a better option and offer us a more accurate representation of our samples' purity.

The antibody used, 6ma, proved to be useful in the spotting of the samples and allowed us to achieve our goal of quantifying the DNA present in them. Despite this, some limitations are present, namely the lack of a negative or positive control and the spotting being done with different amounts of DNA, seeing the samples had different concentrations.

All things considered, we can conclude that the technique developed allowed us to both detect and compare the presence of DNA methylation between different samples and, while some steps can still be taken to further optimize it, can be used for future studying of DNA methylation in the laboratory's samples.

Future perspectives and Shortcomings

The set goal for the future is to apply these optimizations and to study the differences in DNA methylation among different phenotypes, with the expectation that DNA hypermethylation will be observed differently in the two phenotypes of Mecp2 KO mouse model: higher dominance of DNA methylation in the severe model comparing with the mild one. This should be observed together with a decrease in adenosine levels with the consequent decrease of its DNA hypomethilation and anticonvulsant effects. It is also important to address the aspects that could be improved we went over before, seeing they can create possible inaccuracies our results. We thoroughly set ourselves to optimize the studying of the changes in the central nervous system of the mouse model, setting aside the study of the periphery for now, prompting us to consider this strand of study as one of the steps to take in the future. With this knowledge, we can raise the question whether the effects on the methylation and adenosine levels present in the blood are in any way correlated to those happening in the brain. If correlation is found it allows for indirect evaluation through the peripheral levels, making adenosine augmentation strategies easier to execute and manage with the ability to control adenosine levels and consequent avoidance of excessive increases in adenosine.

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