Hugo José Fernandes de Freitas

THE NEUROREGENERATIVE INFLUENCE OF TREADMILL EXERCISE IN STRIATA FROM MICE INTOXICATED WITH METHAMPHETAMINE

Dissertação de Mestrado em Patologia Experimental, apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre, orientada pelos Prof. Doutor Frederico C. Pereira e Prof. Doutor Carlos A. Fontes Ribeiro.

Junho de 2015

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Universidade de Coimbra

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Orientadores: Prof. Doutor Frederico C. Pereira e Prof. Doutor Carlos A. Fontes Ribeiro

Coimbra, 2015

À minha mãe

“Determinação, coragem e autoconfiança são fatores decisivos para o sucesso. Não importa quais sejam os obstáculos e as dificuldades. Se estamos possuídos de uma inabalável determinação, conseguiremos superá-los. Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho.”

Tenzin Gyatso

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A todos vós o meu muito obrigado!

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Publications

Silva CD, Neves AF, Dias AI, **Freitas HJ**, Mendes SM, Pita I, Viana SD, de Oliveira PA, Cunha RA, Fontes Ribeiro CA, Prediger RD, Pereira FC. 2014. A single neurotoxic dose of methamphetamine induces a long-lasting depressive-like behaviour in mice. Neurotox Res. 25(3):295-304.

Abstract

Methamphetamine (METH) is an extremely addictive stimulant drug that produces long-term decreases in dopaminergic (DAergic) markers in the Basal Ganglia (BG), thus remaining an extremely serious health problem.

Physical activity has been documented to have beneficial influence on brain function including neuroprotective/neurorepair properties. More importantly, exercise has been proposed as a treatment for drug addiction.

In this study, we sought to test the hypothesis that treadmill exercise repairs the injured DAergic terminals in the striata from METH-exposed mice.

A total of 24 C57BL/6 mice (12-week-old; 23-26 g) were divided into four groups: two exercised groups were submitted to a seven-week treadmill exercise regimen post-intraperitoneal injection (METH 30mg/kg or NaCl 0.9%; vol. 0.1 ml/10g); the two sedentary groups were also exposed post-intraperitoneal injection to the treadmill but without exercise. The animals were sacrificed 48 hours following the exercise protocol. The striatal dopamine (DA) and its metabolites, DOPAC and HVA, (HPLC-ECD) as well as tyrosine hydroxylase (TH; Western Blotting) were determined as monoaminergic terminal markers. Glial fibrillary acidic protein (GFAP; Western Blotting) density was also measured as a marker of injury-induced astrogliosis. All values were expressed as means ± SEM and statistical significance for multiple comparisons was evaluated by two-way ANOVA and Bonferroni post-tests (treatment x exercise), and the unpaired Student's t-test was used to compare two independent groups (treatment or exercise) (GraphPad Prism 5.00.288). Differences were considered significant at $P < 0.05$.

METH imposed a significant decrease in DA and its metabolites and in TH in the Sedentary group ($P < 0.05$) but not in the Exercised group. This study is suggestive that exercise is provided with regenerative properties to DAergic striatal terminals. However, the mechanism responsible for this positive effect warrants further scrutiny. METH did not change GFAP levels in the Sedentary group. Moreover exercise did not impact GFAP

levels either. However, GFAP levels in the Exercised-METH (EM) group were significantly lower compared to the Sedentary-METH (SM) and Exercised-Saline (ES) groups ($P < 0.05$). The physiological relevance of this finding warrants investigation.

Although these promising results call for further confirmation, one might suggest that treadmill exercise has therapeutic potential on neuronal repair in METH abuse situations.

Key words: *neurotoxicity, methamphetamine, treadmill exercise, neuroregenerative, striata.*

Resumo

A Metanfetamina (METH) é uma droga estimulante muito viciante que produz toxicidade nos terminais dopaminérgicos (DAérgicos) dos Núcleos da Base, representando deste modo um grave problema de saúde.

Têm sido referidas as propriedades neuroprotetoras/reparadoras do exercício físico. Este tem sido particularmente proposto como forma de tratamento para a dependência de drogas.

Neste estudo procurou-se demonstrar a hipótese do exercício (tapete rolante) poder reparar as lesões dos terminais DAérgicos estriatais nos murganhos expostos a METH.

24 murganhos C57BL/6 (de 12 semanas de idade; 23-26g) foram divididos em quatro grupos: dois grupos de exercício foram submetidos a sete semanas de exercício em tapete rolante após uma administração intraperitoneal (METH 30mg/kg ou NaCl 0.9%; vol. 0.1 ml/10g); os dois grupos sedentários foram também administrados intraperitonealmente e expostos ao ambiente do tapete rolante mas sem serem submetidos ao protocolo do exercício. Os animais foram sacrificados 48 horas após o protocolo do exercício. Os níveis estriatais de dopamina (DA) e dos seus metabolitos, DOPAC e HVA, (HPLC-ECD), bem como os níveis da tirosina hidroxilase (TH; Western Blotting) foram determinados como marcadores dos terminais DAérgicos. Os níveis da proteína glial fibrilar ácida (GFAP; Western Blotting) também foram avaliados como marcadores de astrogliose induzida por lesão. Todos os valores foram expressos como média ± SEM. A análise da significância estatística para comparações múltiplas foi calculada através de *two-way ANOVA* e *Bonferroni post-tests* (tratamento x exercício); o *unpaired Student's t-test* foi utilizado para comparar dois grupos independentes (tratamento ou exercício), (GraphPad Prism 5.00.288). As diferenças foram consideradas significativas para $P < 0.05$.

A METH provocou uma diminuição significativa na DA e seus metabolitos e na TH no grupo Sedentário ($P < 0,05$). No entanto, o grupo do Exercício submetido a METH tem os valores dos marcadores DAérgicos normais. Este estudo sugere que o exercício é provido

de propriedades regenerativas para os terminais DAérgicos estriatais. Contudo, o mecanismo responsável por este efeito positivo permanece desconhecido. A METH não alterou os níveis de GFAP no grupo Sedentário. Também o Exercício não produziu modificações nos níveis de GFAP. No entanto, os níveis estriatais de GFAP no grupo Exercício-METH foram significativamente mais baixos em comparação com os grupos Sedentário-METH e Exercício-Salino ($P < 0,05$). A relevância fisiológica deste resultado requer mais estudo.

Apesar destes resultados promissores necessitarem de replicação, podemos sugerir que o exercício em tapete rolante tem potencial terapêutico para produzir reparação neuronal em situações de abuso de METH.

Palavras-chave: *neurotoxicidade, metanfetamina, exercício em tapete rolante, neuroregeneração, estriado*.

Abbreviations

AA	Angstrom
AADC	Aromatic L-amino acid decarboxylase
AC	Adenylate cyclase
ac	Anterior commissure
Acb	Nucleus accumbens
ADHD	Attention deficit hyperactivity disorder
AMPH	Amphetamine
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BG	Basal ganglia
BH-4	Tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CLAP	Chymostatin, leupeptin, antipain, and pepstatin
CNS	Central nervous system
COMT	Catechol-O-methyl-transferase
CPu	Putamen complex
Cyp2D	Cytochrome P450 2D
D1	Dopamine D1 receptor
D2	Dopamine D2 receptor
DA	Dopamine
DAergic	Dopaminergic
DAT	Dopamine transporter
DOPA	Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic acid
DTT	Dithiothreitol

E	Epinephrine
ECF	Chemifluorescence
EGTA	Ethylene glycol tetraacetic acid
EM	Exercised METH
ES	Exercised Saline
GABA	Gamma-aminobutyric acid
GABAergic	Associated to the GABA system
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNFr	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GPe	Globus pallidus externus
GPi	Globus pallidus internus
HPLC-ECD	High-performance liquid chromatography with electrochemical detection
HVA	Homovanillic acid
IGF	Insulin-like growth factor
IGR	Ionotropic glutamate receptor
IP	Intraperitoneal injection
MAO	Monoamine oxidase
METH	Methamphetamine
METH-HCl	Methamphetamine hydrochloride
MNDA	3,4-Methylenedioxy-methamphetamine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSNs	Medium spiny neurons
NE	Norepinephrine
NO	Nitric oxide
O_2	Oxygen molecule
OT	Olfactory tubercle
PBS-T	Phosphate buffer saline with 0.1 % Tween-20
PD	Parkinson’s disease
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene difluoride transfer membrane
RIPA	Radioimmunoprecipitation assay

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	PSodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SM	Sedentary METH
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SS	Sedentary Saline
TH	Tyrosine hydroxylase
t_{max}	Time taken to reach the maximum concentration
USA	United States of America
V*d*	Volume of distribution
VMAT-2	Vesicular monoamine transporter 2
Vol.	Volume

CHAPTER 1

Introduction

Introduction

Methamphetamine (METH) is an extremely addictive stimulant drug (NIDA, 2014) that remains an extremely serious health problem. It is probably the most widely consumed synthetic stimulant in the world. In many countries it is reportedly the second most prevalent illicit drug after cannabis (EMCDDA, 2009).

METH abuse is detrimental for the individual psychologically, medically, and socially. It can cause memory loss, aggression, psychotic behavior, damage to the cardiovascular system, malnutrition, and severe dental problems. METH abuse has also contributed to the increased transmission of infectious diseases, such as hepatitis and HIV/AIDS (Volkow, 2013).

METH deregulates dopaminergic (DAergic) transmission in the brain. Dopamine (DA) is involved in reward, motivation, the experience of pleasure, and motor function. METH's ability to release DA rapidly in reward regions of the brain produces the euphoric "rush" or "flash" that many users experience and can easily lead to addiction, (NIDA, 2014). METH induces damage to DAergic neurons resulting mostly in striatal DA depletion (Riddle et al., 2006; Gouzoulis-Mayfrank and Daumann, 2009).

Beyond METH devastating effects on individual health, its abuse threatens whole communities, causing new waves of crime, unemployment, child neglect or abuse, and other social ills. For example, METH abuse in the United States of America (USA) costed approximately $23.4 billion, around 21.3 thousand millions Euros, in 2005 (Volkow, 2013).

In this study we propose a neuroregenerative therapy for METH abuse using the treadmill exercise. The main goal is to evaluate the effect of treadmill exercise on mice striatal DAergic METH-induced dysfunction.

In this document, it is firstly presented an introductory part describing the METH characteristics, the neuroanatomophysiology of the striata - major brain target of METH neurotoxicity, and the neuroregenerative effects of exercise. Secondly, the aim of this

study, the materials and methods, and the results are presented in the third and fourth chapters, respectively. The discussion and the conclusion are presented in the fifth and in the sixth chapters, respectively

1.1. Methamphetamine

METH (Figure 1) is a central nervous system (CNS) stimulant that can be used for medical and for recreational purposes (U.S. Food and Drug Administration, 2013).

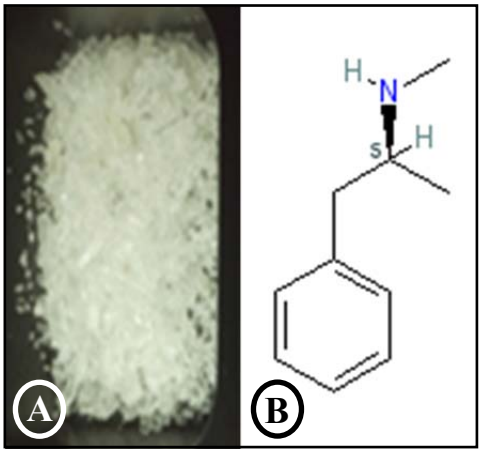

Figure 1. - **A -** METH picture. Image provided by the UK National and taken from EMCDDA, 2014; **B -** METH 2D structure. Image taken from IUPHAR/BPS, 2014.

METH is an amphetamine (AMPH) derivative with an increased CNS penetration and a longer half-life, and its effects may persist for 6 to 24 hours longer compared to AMPH. METH can be ingested orally, smoked, or snorted and produces more CNS stimulation with less peripheral effects compared to AMPH. However, large doses may result in hypertension and ischeamic or hemorrhagic stroke (Karch, 1998; Karch, 2008).

METH is a stereoisomer drug and is available in two forms: the D- and L. The D-form has greater central stimulant activity than the L-isomer, which has greater peripheral sympathomimetic activity. The D-METH is commonly used as a drug of abuse while the L-isomer was normally used in non-prescription inhalers as a decongestant.

1.1.1. Medical use

METH was prescribed for many years to treat narcolepsy, at some time it was sold without a prescription as an appetite suppressant. Nowadays its availability in the form of prescription drug is under the close scrutiny of a physician (Karch, 1998). For example, desoxyn® (METH) is a CNS stimulant prescription medicine used for the treatment of Attention-Deficit Hyperactivity Disorder (ADHD). METH may help increase attention and decrease impulsiveness and hyperactivity in patients with ADHD. It is also used short-term, along with a low calorie diet, for weight loss in obese patients who have not been able to lose weight on other therapies (U.S. Food and Drug Administration, 2013).

1.1.2. Illicit use

The clandestine production of METH in the forms of a powder or granular material has been one of the major problems facing law enforcement personnel in the USA (Karch, 1998) and its consumption remains a problematic health problem due to its highly addictive characteristics (EMCDDA, 2009). The illicit METH is synthesized from the precursors phenylacetone and N-methylformamide (DL mixture) or alternatively from ephedrine by red phosphorus/acid reduction (Karch, 1998; Karch, 2008).

The illicit use could lead to acute toxicity situations manifested as rhabdomyolysis, disseminated intravascular coagulation, pulmonary oedema, vascular spasm, acute myocardial infarction and stimulant and psychedelic effects (Karch, 1998).

1.1.3. Absorption

METH is readily absorbed across the gastrointestinal tract with the time taken to reach the maximum concentration (t_{max}) values ranging from 3.13 to 6.3 h post-ingestion (Schep et al., 2010). An oral dose of 5 to 10 mg of METH results in blood concentrations from 20 to 60 ng/mL and a peak METH plasma concentration circa 3.6h (Karch, 1998; Karch, 2008).

After intranasal administration of the powder peak plasma occur approximately 3-4 h post-exposure. The inhalation of the METH vapor rapidly appears in the plasma but plasma concentrations increase slowly, peak concentrations being reached at 2.5 (±0.5) h (Schep et al., 2010).

1.1.4. Distribution

METH has a relatively high lipophilicity and low molecular weight. Therefore METH is distributed to most parts of the body. Reported volumes of distribution of habitual abusers were 3.73 ± 0.94 and 3.80 ± 1.05 L/kg following doses of 0.25 mg/kg and 0.5 mg/kg, respectively. Additionally, it is expected to distribute extensively across high lipid-content tissues such as the blood-brain barrier (BBB), breast milk and maternal to fetal blood (Schep et al., 2010). However, there is limited information on whether METH significantly binds to plasma proteins.

1.1.5. Metabolism and excretion

The predominant site of METH metabolism is the liver, mainly involving CYP2D6 (Schep et al., 2010). The major active metabolite of METH is AMPH no matter the route of administration. The D- and L-forms suffer hydroxylation and N-demethylation to their respective p-hydroxymethamphetamine and AMPH metabolites (Figure 2). Under normal conditions up to 43% of a D-METH dose is excreted unchanged in the urine in the first 24 hours and 4 to 7% will be present as AMPH. METH is a weak base (pKa ∼9.9) (Schep et al., 2010). Therefore, up to 76% of METH is present as parent drug in acidic urine compared with 2% under alkaline conditions (Karch, 1998; Karch, 2008).

L-METH is biotransformed similarly to the D-isomer but at a slower rate. D-METH is commonly self-administered by the smoked route with a concentrations plateauing (40 to 44 ng/ml) after 1 h. Then concentrations in plasma decline slowly, reaching the same concentration at 8 h on the downward side of the curve as reached at 30 min on the upward side. The average elimination half-life is 11.7h with a range from 8 to 17h. The route of administration imposes different subjective effects. For example, smoking induces a greater “high” when compare to oral METH (Karch, 1998; Karch, 2008).

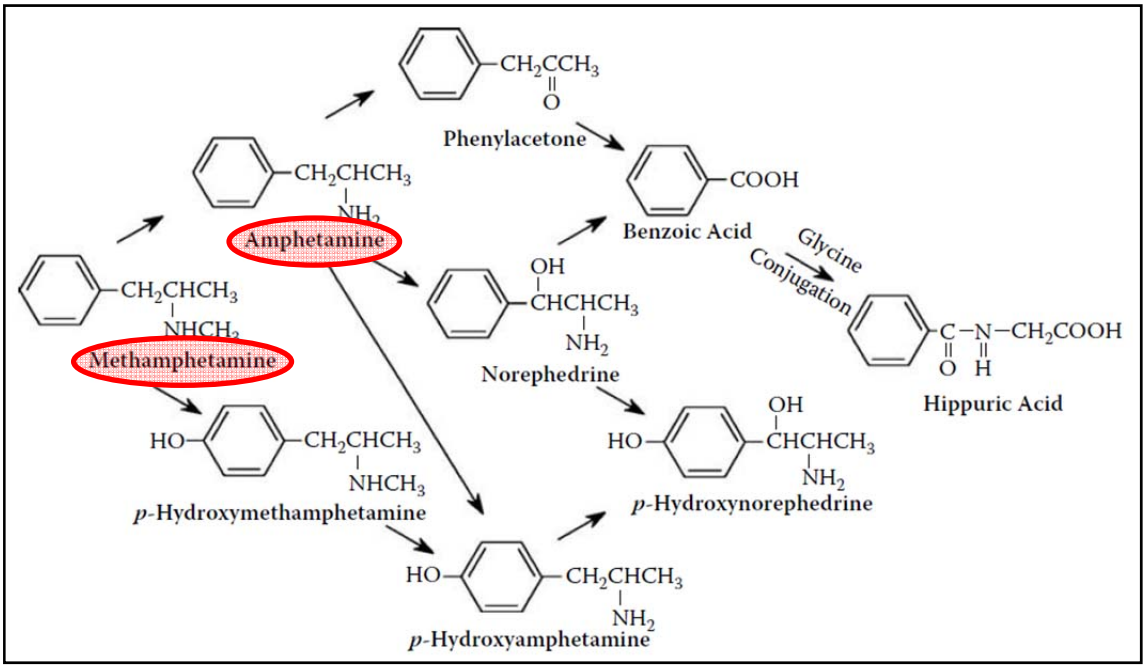

Figure 2. Metabolic pathway of AMPH and METH. Image taken from Karch, 2008.

1.2. Striatum

Basal ganglia (BG) play an important role in the control of posture and voluntary movement and have no direct input from or output connections with the spinal cord (Snell, 2010). The striatum is the largest structure of the BG, receiving synaptic input from multiple regions including the neocortex, thalamus, external globus pallidus (GPe), and the substantia nigra pars compacta (SNc) of the midbrain. Striatum is generally implicated in movement regulation and has been increasingly linked to cognitive functions. (Hiebert et al., 2014). Additionally, there is evidence that striatum participates in the transition from casual to habitual drug use (Silva et al., 2014).

Striatum could be subdivided in the caudate-putamen complex (CPu), the nucleus accumbens (Acb) and the striatal elements of the olfactory tubercle (OT) (Voorn et al., 2004).

Classically the striatum consists of a dorsal sensorimotor part and a ventral portion processing limbic information. But anatomy and neurophysiology show that the two striatal areas have the same basic structure and that sharp boundaries are absent. There is

evidence that a distinction between dorsolateral and ventromedial seems most valid, in accordance with a mediolateral functional zonation imposed on the striatum by its excitatory cortical, thalamic and amygdaloid inputs (Voorn et al., 2004). Therefore, the more mediolateral-oriented functional striatal gradient has been highlighted.

Some studies argue that there is a border between the nucleus accumbens and caudate-putamen complex (white dashed line in Figure 3) but without a clear histological or immunohistochemical basis. Another subdivision adopted is a straight line from the inferior tip of the lateral ventricle medially to the most medial extension of the external capsule laterally, forming an imaginary boundary between dorsal and ventral striatum (straight border between orange and gray zones in Figure 3) (Voorn et al., 2004). The striatal complex receives glutamatergic excitatory inputs from cortical and thalamic structures and DAergic inputs from SNc. These dual glutamatergic and DAergic projections converge onto dendritic spines of the GABAergic output medium spiny neurons (MSNs) that represent about 95% of striatal neurons. Moreover, striatal interneurons comprising spiny GABAergic and large cholinergic interneurons receive also these inputs, and most of them synapse onto MSNs (Calabresi et al., 2014).

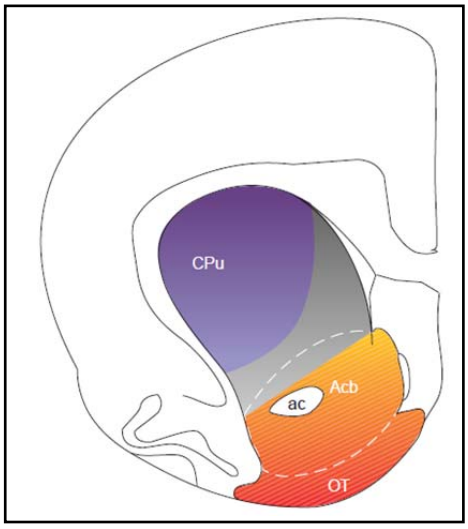

Figure 3. Transverse section of the rat forebrain showing the striatum. CPu, caudate-putamen complex; Acb, nucleus accumbens; OT, olfactory tubercle; ac, anterior commissure. Image taken from Voorn et al., 2004.

1.2.1. Direct and indirect pathways of basal ganglia

The BG is functionally dependent upon two circuits, the direct and indirect pathways, that originate from distinct populations of striatal MSNs and project to different output structures, including globus pallidus and substantia nigra pars reticulata (SNr) (Figure 4). Recently, it was proposed that these pathways are structurally and functionally intertwined. Thus, all MSNs might either facilitate or inhibit movement depending on the form of synaptic plasticity expressed at a certain moment (Calabresi et al., 2014). DA arising from the SNc activates D1-expressing striatal MSNs of the direct pathway and inhibits D2-expressing striatal neurons of the indirect pathway. The output nuclei globus pallidus internus (GPi) and SNr project to the thalamus, which in turn sends efferents that complete the cortico-basal ganglia-thalamo-cortical loop (Figure 4, Calabresi et al., 2014)

While D1 receptors activate the $G\alpha_{s/olf}$ family of G proteins to stimulate the cyclic adenosine monophosphate (cAMP) production, D2 receptors act through $G\alpha_{i/o}$ family of G proteins and thus induce a decrease in cAMP levels (Purves et al., 2008).

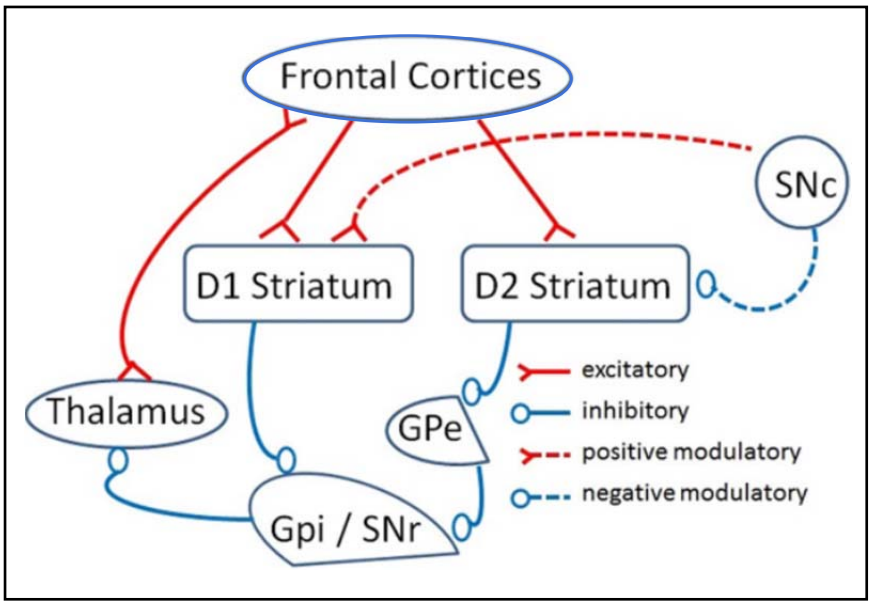

Figure 4. Schematic representation of direct/indirect pathway of the basal ganglia. DA arising from the SNc activates D1-expressing striatal MSNs of the direct pathway and inhibits D2-expressing striatal neurons of the indirect pathway. SNc, Substantia nigra pars compacta; SNr, substantia nigra pars reticulata; GPi, globus pallidus internus; GPe, globus pallidus externus. Image taken from Kurniawan et al., 2011.

1.3. The life cycle of dopamine

DA was originally synthesized in 1910 before its importance as a neurotransmitter was acknowledged (Meiser et al., 2013). The first time DA was found to occur in an organism was as a pigment-building metabolite in the plant *Sarothamnus scoparius*. Later it was found to be a substrate of aromatic amino acid decarboxylase (AADC), which could be isolated from sympathetic ganglia and other animal tissues. Initially DA was only assumed to be a precursor of the catecholic neurotransmitters epinephrine (E) and norepinephrine (NE) or considered to be an intermediate in tyrosine degradation. The recognition of DA as an independent neurotransmitter only occurred later and only after that the first DA receptor was discovered.

1.3.1. Dopamine biosynthesis and metabolism

Figure 5 illustrates a nigrostriatal DAergic terminal. The main pathway for DA biosynthesis starts at tyrosine or phenylalanine which can be hydroxylated by phenylalanine hydroxylase (Figure 6). Tyrosine is hydroxylated to form dihydroxyphenylalanine (DOPA) by tetrahydrobiopterin (BH4)-dependent tyrosine hydroxylase (TH) or alternatively by tyrosinase. TH consists of four identical subunits (Figure 7), each catalytically active and each of them requiring BH4, ferrous ion and oxygen molecule (O_2) to oxidize tyrosine to DOPA (Meiser et al., 2013). The structure of all four isoforms is based on the same principle: one N-terminal regulatory domain (~150AA), a central catalytic domain (~300AA) and the C-terminal part, coding for a leucine zipper domain which is responsible for tetramer formation. The loss of tetramer formation ability leads to a 70% drop of TH activity. Moreover, TH is the gold standard marker in the identification of DAergic neurons, because it is the rate limiting enzyme in DA synthesis that catalyze the conversion of L-tyrosine to L-DOPA (White and Thomas, 2012). The decarboxylation of DOPA by AADC leads then to DA. DA can be obtained by other biosynthesis pathway: tyrosine is transformed into tyramine by the AADC, being then oxidized into DA by cytochrome P450 2D (Cyp2D). The DA degradation is performed by variable ways that mainly ends in DOPAC and HVA (Figure 6) (Meiser et al., 2013).

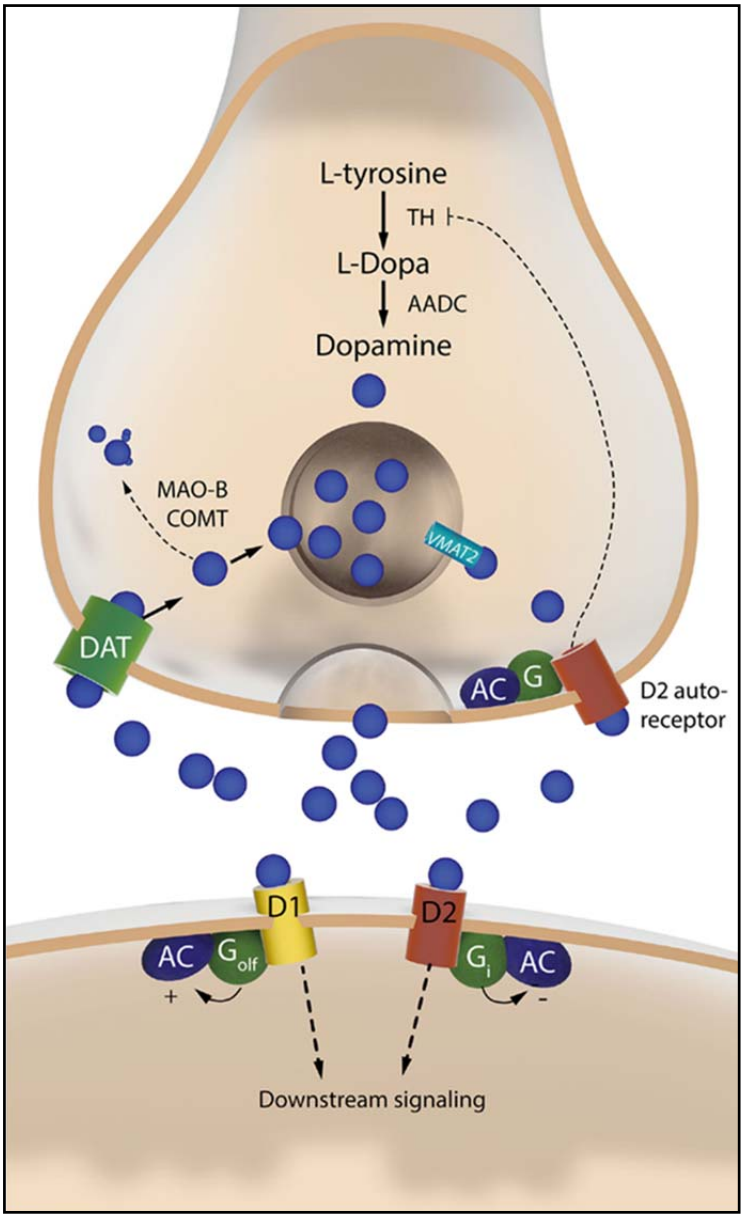

Figure 5. Nigrostriatal dopaminergic terminal. This figure depicts dopamine (DA; blue circles) synthesis; DA transport into DA vesicles through VMAT2 - vesicle monoamine transporter-2; DA release; DA reuptake via DAT dopamine transporter and DA metabolism via MAO-B and COMT. COMT is essentially expressed by glial cells. Post-synaptic dopaminergic signalling is mediated by D1 receptor which is coupled to G_{olf} and by D2 receptor coupled to G_i. AC is activated by D1R and inhibited by D2R. D2R autoreceptor inhibits DA synthesis. AADC, aromatic L-amino acid decarboxylase; AC, adenylate cyclase; COMT, catechol-O-methyl-transferase; DAT, dopamine transporter; MAO-B, monoamine oxidase B; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2. Image taken from Cenci, 2014.

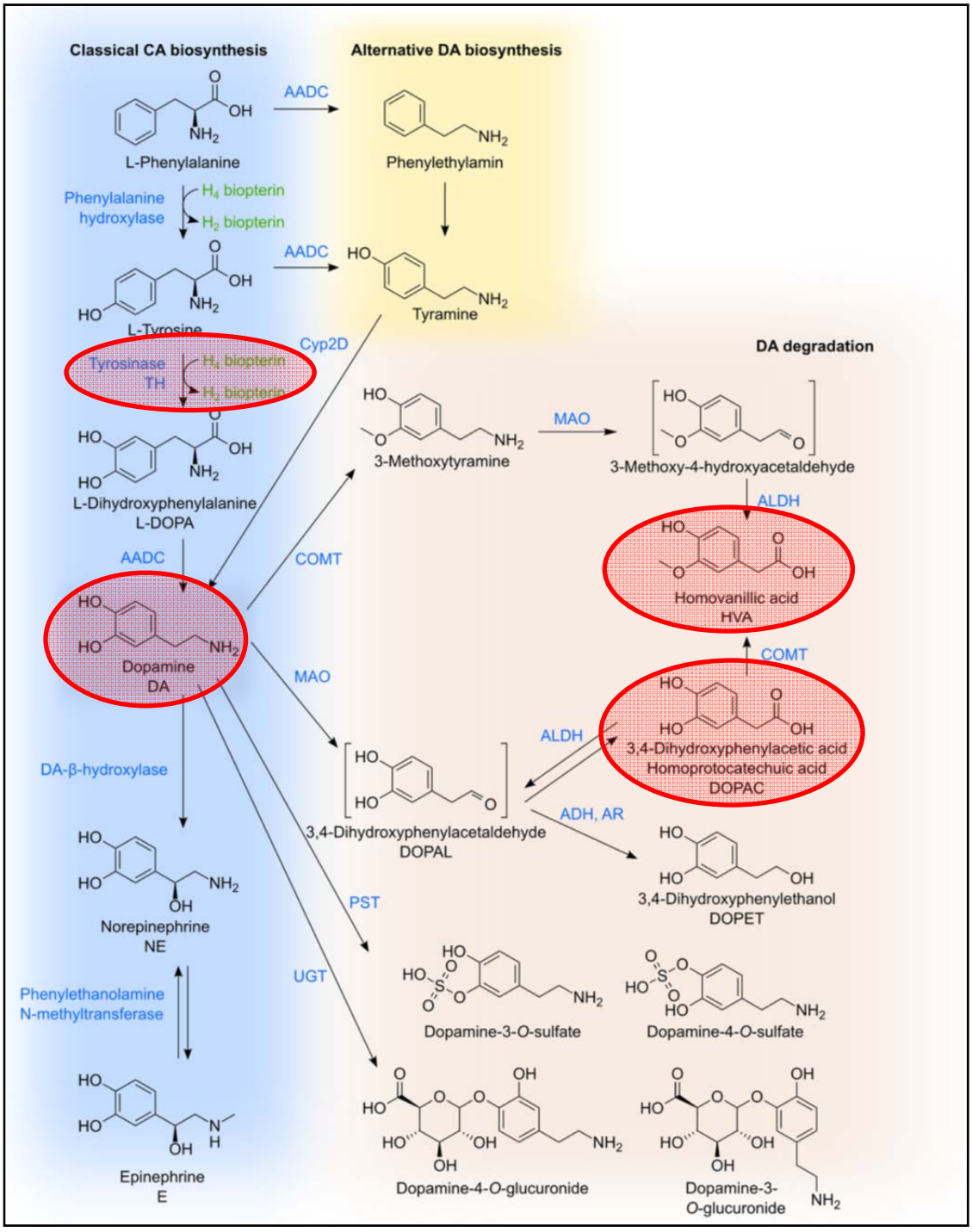

Figure 6. Dopamine biosynthesis and degradation. - Markers of DA biosynthesis and degradation assessed in this study. Image taken from Meiser et al., 2013.

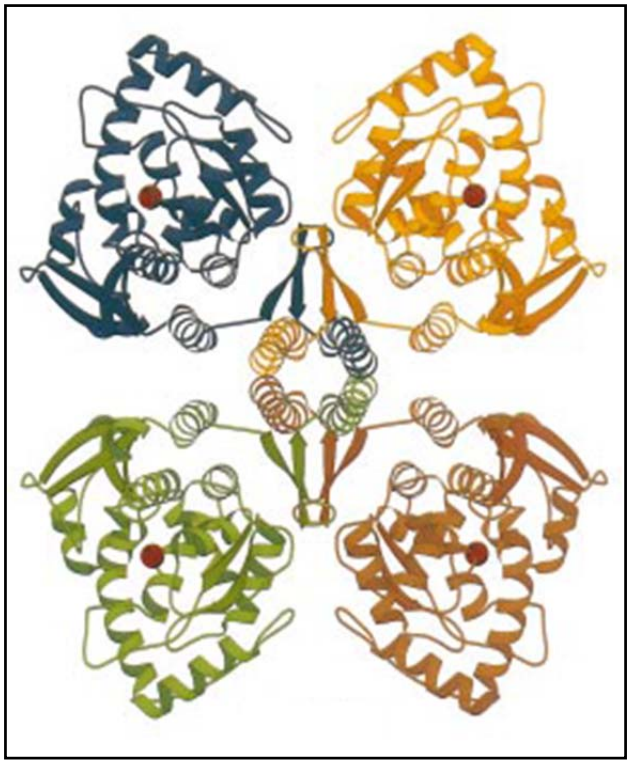

Figure 7. Ribbon diagram representations of the TH tetramers; the four subunits are related by a crystallographic 222 symmetry. Image taken from Fusetti et al., 1998.

1.4. Methamphetamine induced neurotoxicity

METH can lead to detrimental psychological, cardiovascular, and other systemic effects, and, following long-term abuse, neuronal apoptosis, and nerve terminal degeneration (Schep et al., 2010).

1.4.1. Dopaminergic neurotoxicity

METH triggers an aberrant release of DA from the presynaptic terminal into the synaptic cleft. This is the outcome of the primary mechanisms of action of METH: the redistribution of DA from synaptic vesicles to the cytosol through the inhibition of vesicular monoamine transporter 2 (VMAT-2) and the reverse transport of DA via dopamine transporter (DAT) (Pereira et al., 2004, 2006, 2011). Additionally, METH has been shown to block the activity of DAT. Continuous use of METH may lead to long-term damage to striatal DAergic terminals trough a wealth of intertwined

mechanism that are depicted in figure 8 (Riddle et al., 2006; Pereira et al., 2004, 2006, 2011; Yamamoto et al., 2010) and are as follows:

(1) Inhibition of mitochondrial function leading to decreased cellular energy and increased reactive species levels (reactive oxygen species - ROS)

(2) Decrease in DAT activity producing cytosolic DA build up and fostering reactive ROS formation;

(3) Alterations in VMAT-2 activity and trafficking contributing to DA build up and ROS formation via auto-oxidation of DA;

(4) D1R involvement in ROS formation, N-methyl-D-aspartate (NMDA) receptor signalling, and DAT function;

(5) D2R involvement in VMAT-2 trafficking and glutamate receptor signalling;

(6) METH induced hyperthermia;

(7) Ionotropic glutamate receptor (IGR) induced Ca^{2+} influx and subsequent mitochondrial damage and ROS formation, as well as NMDA receptor mediated nitric oxide (NO) production;

(8) Glial activation, neuroinflammation, oxidative stress, BBB maintenance.

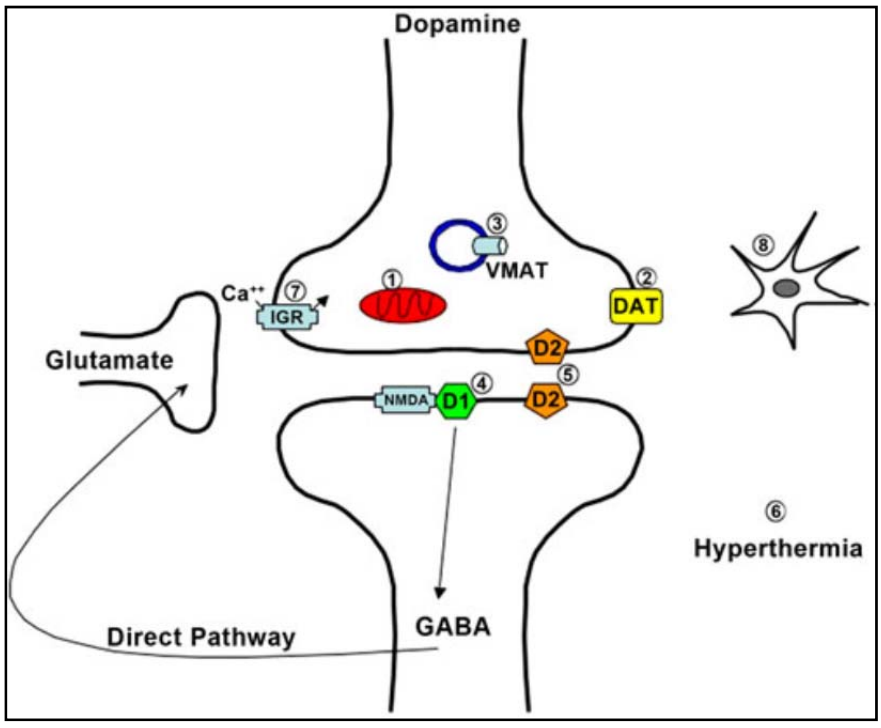

Figure 8. Components of METH induced DAergic neurodegeneration. (1) mitochondria; (2) DAT, dopamine transporter; (3) VMAT, vesicular monoamine transporter-2; (4) D1, dopamine D1 receptor; (5) D2, dopamine D2 receptor; (6) hyperthermia; (7) ionotropic glutamate receptor (IGR); and (8) glial cells. Image taken from Riddle et al., 2006.

Those alterations on the DAergic system may translate into striatal DA depletion and lower striatal DAT and TH densities that may persist even after years of abstinence from METH (Gouzoulis-Mayfrank and Daumann, 2009). These DAergic toxicity may be associated with deficits in motor and cognitive performance and psychopathological abnormalities. These METH-induced detrimental effects to DAergic terminals have been reproduced in animals models (Pereira et al., 2004, 2006, 2011; Silva et al., 2014).

1.4.2. Astrogliosis

Astrocytes, or astroglia, are the most abundant cells in the CNS and are classically identified as the cells that express the intermediate filament glial fibrillary acidic protein (GFAP) (Zhang et al., 2010). GFAP is the major intermediate filament protein in mature astrocytes and forms an important part of the intermediate filament cytoskeleton of the astrocyte. Astrocytes contribute to several functions that are described in Figure 9 and include support during CNS development, ion homeostasis, neurotransmitter uptake, metabolic support, synapse function, the maintenance of BBB integrity, contribution to the CNS, immune system and neuromodulation (Kitamura et al., 2010). Astrocytes have also an important role in regulating the function of oligodendrocytes and neural stem cells.

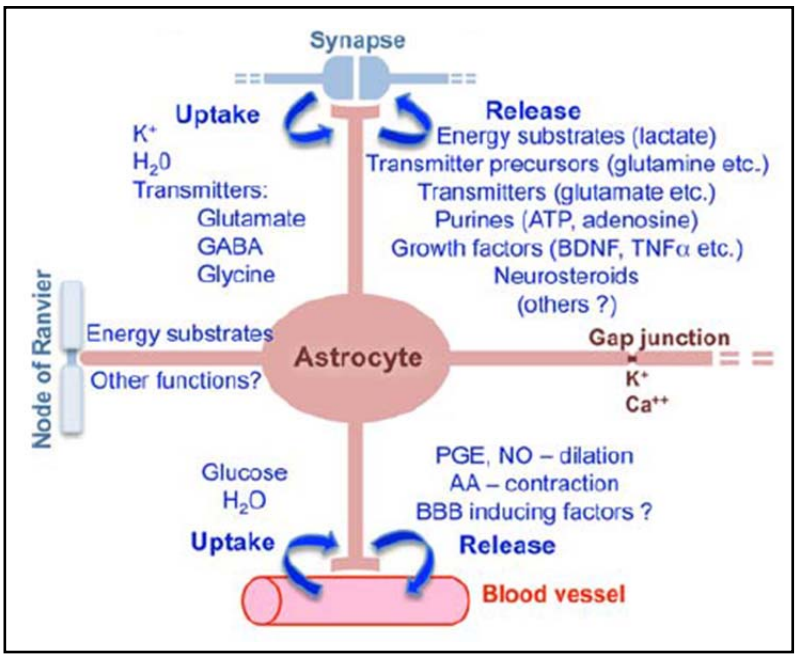

Figure 9. Astrocyte physiology in healthy CNS. Image taken from Sofroniew and Vinters, 2010.

Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis (Figure 10), which has become a pathological hallmark of CNS structural lesions (Sofroniew and Vinters, 2010). In fact, astrogliosis is induced in various kinds of pathological conditions such as neurodegenerative diseases, ischemia or trauma, and is considered to be one reliable marker for neuronal injury. Astrogliosis is characterized by the increase of intermediate filaments with accompanying cellular hypertrophy and an abnormal apparent increase in the number of astrocytes. For example, upregulation of intermediate filament proteins like GFAP by astrocytes is regarded as the hallmark of astrogliosis (Zhang et al., 2010).

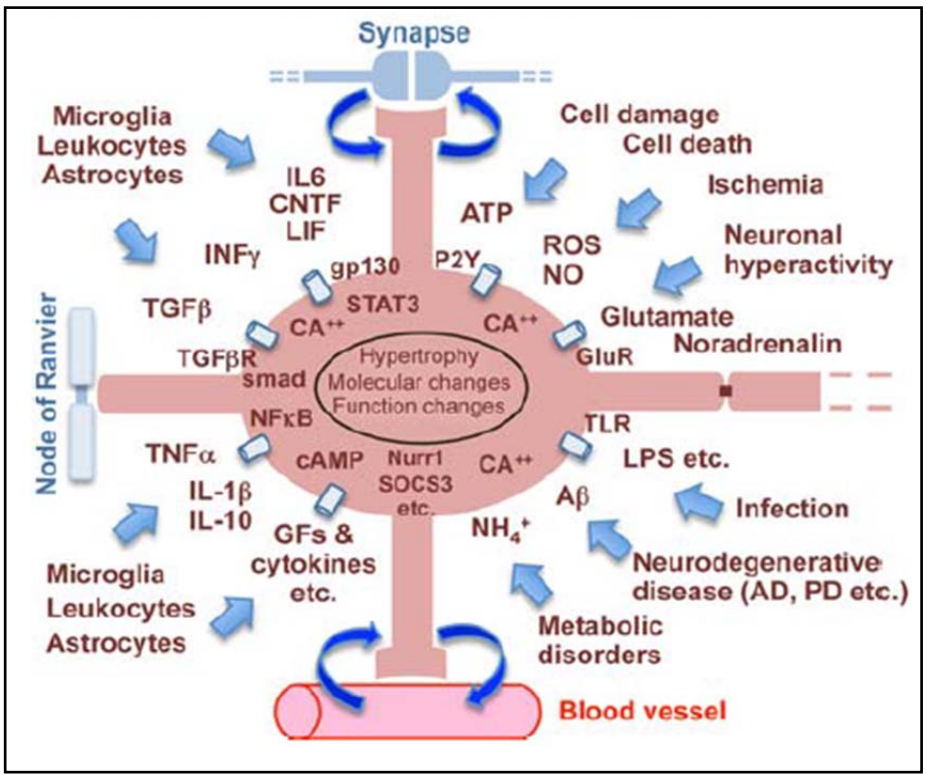

Figure 10. Molecular regulators of reactive astrogliosis. Image taken from Sofroniew and Vinters, 2010.

Astrocytes are also sensitive to toxicant-induced damage in the CNS and it has been demonstrated *in vitro* and *in vivo* that METH increases the GFAP levels and lead to astrogliosis. Therefore, GFAP expression has been suggested as a marker for METH-induced neurotoxicity (Kitamura et al., 2010).

However, the mechanisms of METH-induced neurotoxicity are not fully understood and animal models including mice model used herein are certainly instrumental to shed further light on this issue.

1.5. Neuroregenerative effects of exercise

Multidisciplinary literature has documented the beneficial influence of physical activity including aerobic exercise on selective aspects of brain function (Hillman et al., 2008). In fact, there is a growing body of preclinical and clinical evidence showing that physical exercise has broad effects on overall brain health, including benefits in learning, memory capacity, neuroprotective capability and alleviation of depression (Cotman et al., 2007).

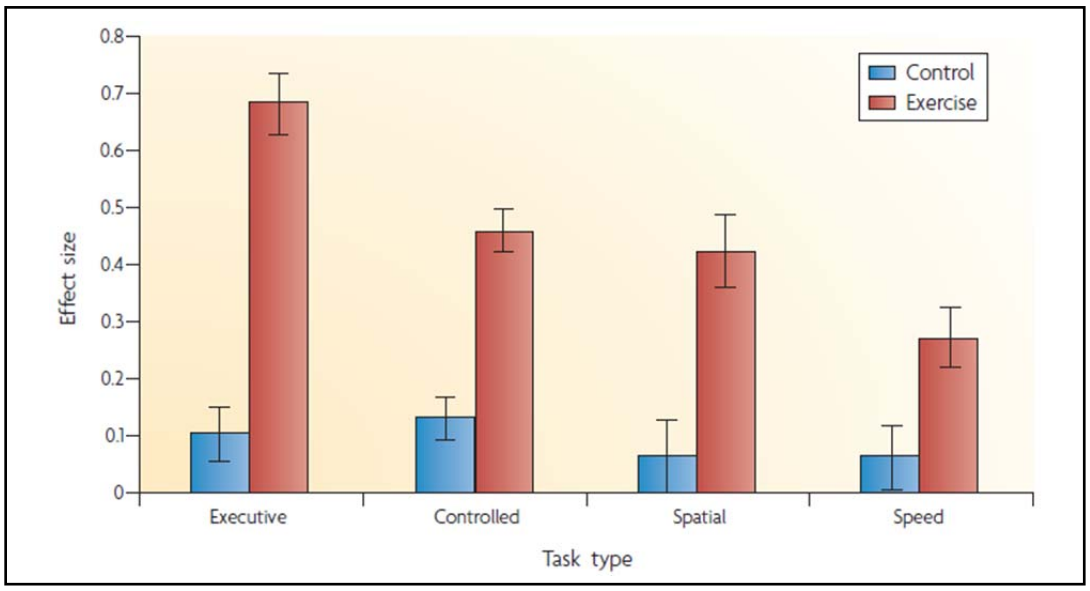

Figure 11. Meta-analytic findings of exercise-training effects on cognition in older adults. The results of a meta-analysis of the effects of fitness training on cognition showed that the benefits of fitness training on four different cognitive tasks were significant. Image taken from Hillman et al., 2008.

For example, there is evidence that aerobic fitness training shows benefits on cognitive tasks, in particular executive and control processes, in older adults (Figure 11) (Hillman et al., 2008). Additionally, aerobic exercise increases brain volume, both gray and white matter regions, in aging humans (Colcombe et al., 2006). Preclinical studies have shown

that chronic aerobic exercise can lead to the growth of new capillaries in the brain and increase the length and number of the dendritic interconnections between neurons. Aerobic exercise can also induce neurogenesis and strengthen metabolism (Colcombe et al., 2006). Those properties are likely to be driven by increases in growth factors such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) that results in structural changes that better interconnect the brain, thus increasing its plasticity. The exercise is also associated with the reduction of risk factors such as diabetes, hypertension and cardiovascular disease, which converge to cause brain dysfunction and neurodegeneration (Cotman et al., 2007).

In particular, there are solid data on exercise modulating BG function. For example, there is evidence supporting the potential effect of exercise in modifying synaptic connectivity within the DA-depleted striatum in Parkinson´s disease (PD) models (Toy et al., 2014). Exercise is being currently proposed as a novel strategy aiming to modify the progression of PD possibly through neuroprotective mechanisms (Earhart and Falvo, 2013). The increase of DA signalling by exercise may be justified through the increased availability of neurotrophic factors, which in turn can promote mitochondrial energy production, antioxidant defense, synaptogenesis, reduced inflammation, angiogenesis, and other processes that suppress apoptosis (Figure 12). Exercise reduces the long-term behavioural effects of DA-directed neurotoxins in animal models and could support normal DA signaling that compensates for non-functional DA neurons (Figure 12) (Zigmond and Smeyne, 2014). Recently, it was argued that a structured exercise program including aerobic treadmill and strength exercise is an effective intervention for improving symptoms of depression and anxiety associated with METH abstinence in addicts (Rawson et al., 2015).

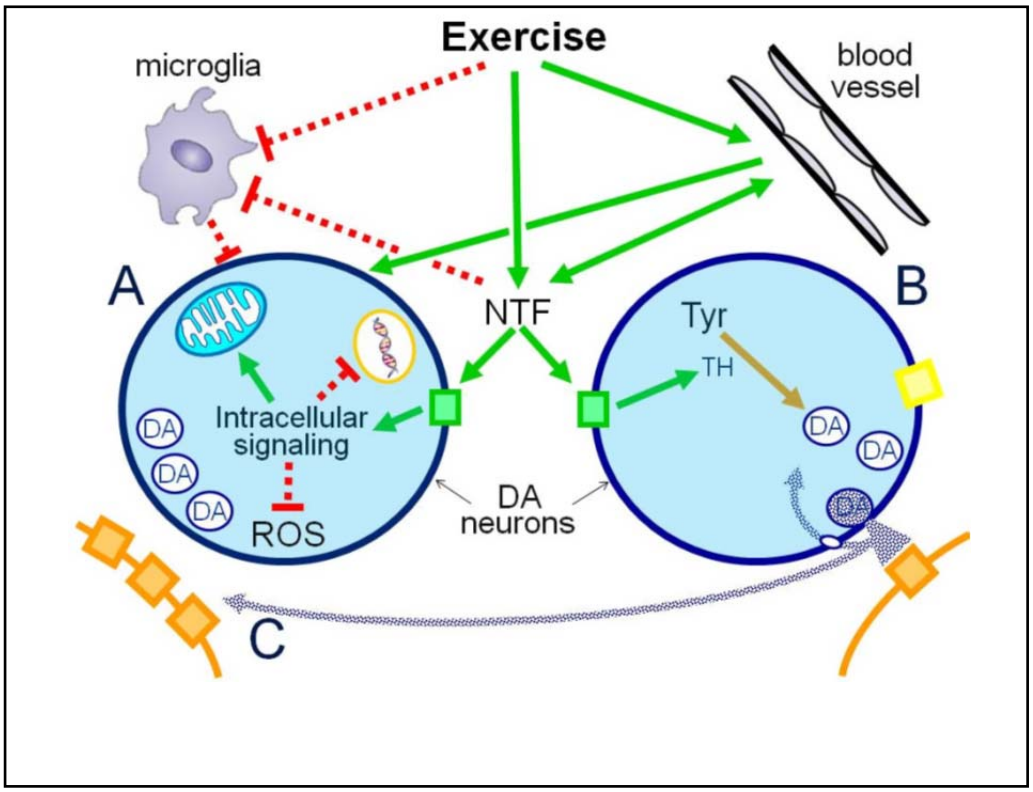

Figure 12. Model for exercise-induced protection against toxin-induced loss of DA function. (A) On the left is shown a DA neuron being protected or rescued by exercise. (B) On the right is shown exercise increasing the capacity of otherwise healthy DA neurons to deliver a signal by increasing DA synthesis via increased TH activity, increasing DA release, and decreasing DA reuptake. (C) This can compensate for non-functional neurons (left) via the combination of increased DA diffusion and increased target sensitivity to DA due to the absence of presynaptic DA uptake sites and increased postsynaptic receptors. Solid arrows indicate an excitatory effect; dotted lines indicate inhibition. Image taken from Zigmond and Smeyne, 2014).

CHAPTER 2

Aims

Aims of the present study

The main goal of this study was to test the hypothesis that treadmill exercise is endowed with neuroregenerative properties on striatal DAergic system after METH-induced dopaminergic dysfunction.

CHAPTHER 3

Materials and methods

3.1. Animal care and use

Robust evidence showed that C57BL mice were sensitive to METH-induced striatal DA depletions (Karsnova and Cadet, 2009) and were useful in studies that used treadmill exercise (Lau et al., 2011; Petzinger, 2009; Al-Jarrah et al., 2013).

Figure 13. Male mice C57BL/6J 12 weeks old. Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra.

In this study, we used 24 male mice C57BL/6J 12 weeks old, weighing 23 to 26 g (Figure 13) from Charles River Laboratories Inc. (Barcelona-Spain). Animals were randomly assigned to four groups as shown in Table 2.

Table 1. Experimental groups

Group n=6	IP injection	Sedentary	Exercised
SS	Saline	+	-
SM	METH	+	-
ES	Saline	-	+
EM	METH	-	+
SS - Sedentary Saline; SM - Sedentary METH; ES - Exercised Saline; EM - Exercised METH; (+) - Group involved; (-) - Group not involved			

Mice were housed in groups of six per cage with free access to food and water, controlled conditions of temperature and humidity and maintained on a 12 h light/dark cycle (lights

on at 08:00) in the *vivarium* of the Faculty of Medicine the University of Coimbra. The ARRIVE guidelines have been followed. Consistently, attempts were made to minimize the number of animals used and their suffering. The animal procedures were performed in accordance with the ‘‘Guide for the Care and Use of Laboratory Animals’’ (Institute of Laboratory Animal Resources, National Academy Press, 1996).

All experiments were approved by the Institutional Animal Care and Use Committee from Faculty of Medicine, Coimbra University, and were performed following the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Figure 14 shows the experimental design followed in this study.

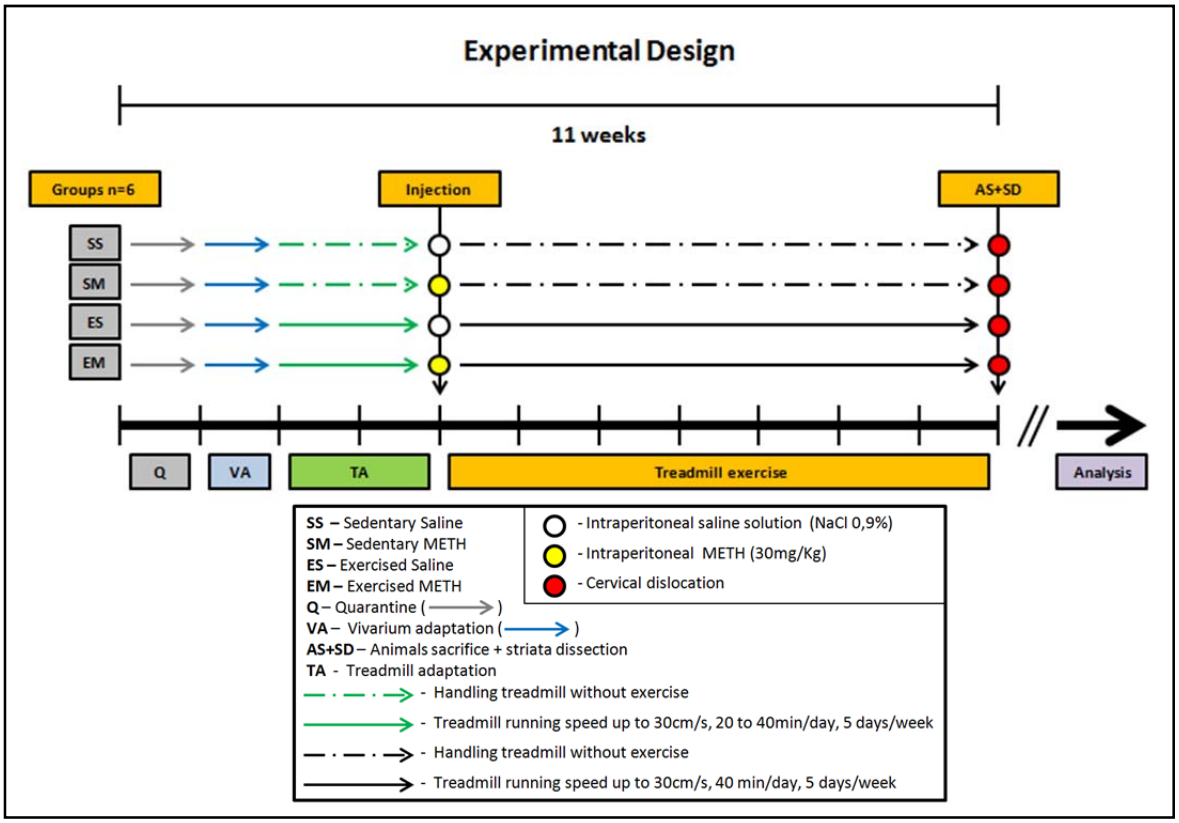

Figure 14. Experimental design used in this study

Mice were killed by cervical dislocation and brains were rapidly removed and dissected on ice, at the end of the experimental protocol. Striata were stored at -80°C until high pressure liquid chromatography with electrochemical detection (HPLC-ECD) and

Western-blot analyses. Left striata were used for protein (TH and GFAP) analysis by Western-blot, whereas right striata were used for determination of monoamine (DA, DOPAC and HVA) contents by HPLC-ECD.

3.2. Meth preparation and treatment

We were issued permission to import METH-HCl from Sigma-Aldrich (St. Louis, MO, USA) by INFARMED, Portugal (National Authority of Medicines and Health Products). Animals were injected intraperitoneally with a single dose of METH (30 mg/kg) or with saline solution (0.9 % NaCl; SAL) in a volume of 0.1 mL/10 g of body weight (Fig. 15). This METH regimen is representative of an acute toxic dosing (ATD); as suggested by Davidson et al. (2001), and extensively discussed by Krasnova and Cadet (2009). This single high dose METH protocol offers greater experimental control over variables and has been successfully used by us (Pereira et al., 2006, Pereira et al., 2012; Silva et al., 2014).

Figure 15. Intraperitoneal injection performed by Pereira F, PhD. Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra

3.3 Treadmill exercise

Treadmill running has been used extensively over the past four decades to study behavioral, physiological, biochemical, and, more recently, molecular responses to both acute exercise stress and chronic exercise training (Kregel et al., 2006). Importantly, there are evidences that forced treadmill exercise induced stronger neuroprotection as compared to other training exercises (Kinni et al., 2011). Additionally this type of exercise provides control of exercise intensity and duration as well as well-defined experimental conditions (Kregel et al., 2006).

The treadmill exercise protocol used herein is based on several previous studies about exercise and neurodegeneration in mice (Al-Jarrah et al., 2007; Pothakos et al., 2009; Thanos et al., 2010; Smith et al., 2011; Fu et al., 2012). The exercise-training protocol started with a two-weeks treadmill adaptation period before METH treatment and continued for seven weeks post-METH injection (9 weeks total) (Figure 16). There was a constant surveillance of the animals while on treadmill to prevent them from being injured (e.g., breaking toenails or injuring their paws) (Kregel et al., 2006).

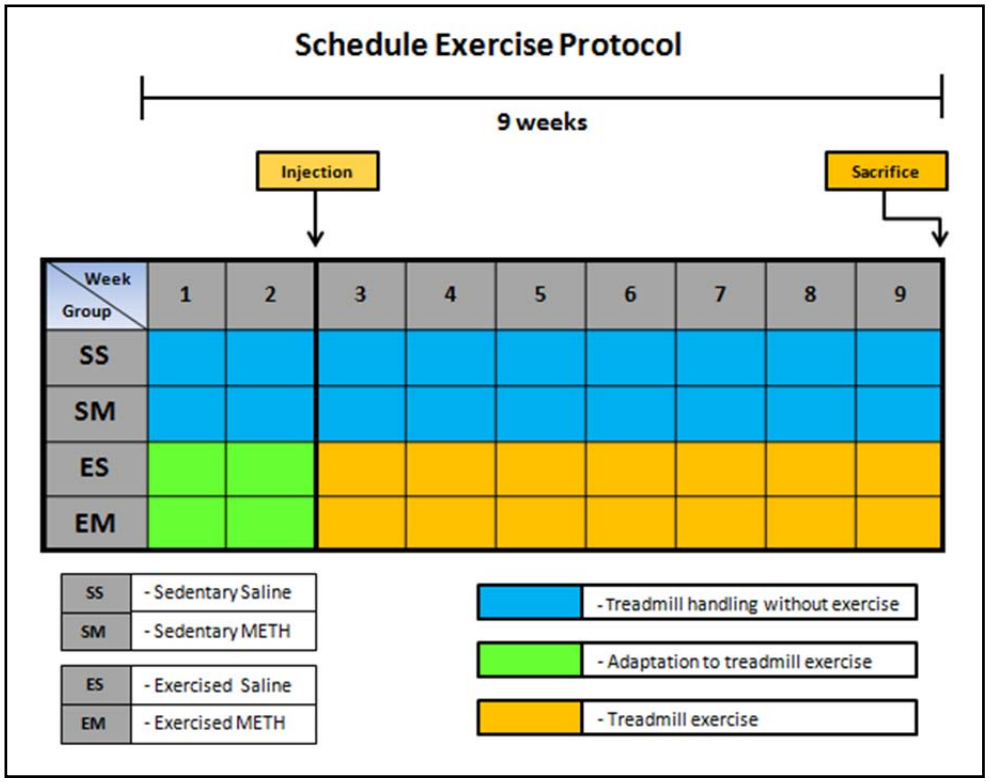

Figure 16. Schedule exercise protocol establish in our laboratory.

The exercised groups were trained in the morning period (9-11h), five days/week on a treadmill with no inclination (Figures 17 and 18) as follows:

First week:

- Day 1-2: 20 min/day (20 cm/s)
- Day 3-5: 30 min/day (20cm/s, 5 min + 25cm/s, 20 min + 20cm/s, 5 min)

Second week:

- Day 8-10: 30 min/day (20cm/s, 5 min + 30cm/s, 20 min + 20cm/s, 5 min)
- Day 11-12: 40 min/day (20cm/s, 5 min + 30cm/s, 30 min + 20cm/s 5 min)

Third to ninth weeks:

- 40 min/day (20cm/s, 5 min + 30cm/s, 30 min + 20cm/s 5 min)

The treadmill exercise was performed in a four-lane treadmill adapted for mice (Panlab/Letica LE8706, Barcelona, Spain) as shown in Figure 17 and a two-lane treadmill adapted for mice (Panlab/Letica LE8700, Barcelona, Spain).

Figure 17. Treadmill exercise. **A** - outside view; **B** - inside view. Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra.

Before METH treatment, the exercised animals were introduced to the treadmill slowly over the course of two weeks with initial orientation and guidance on the moving treadmill. The METH treatment was made after mice could run 40 min/day at a speed of 30 cm/s (Figure 18). Sedentary mice were not exercised. However, they were transported to the training room so that they could experience the treadmill conditions (Figure 18).

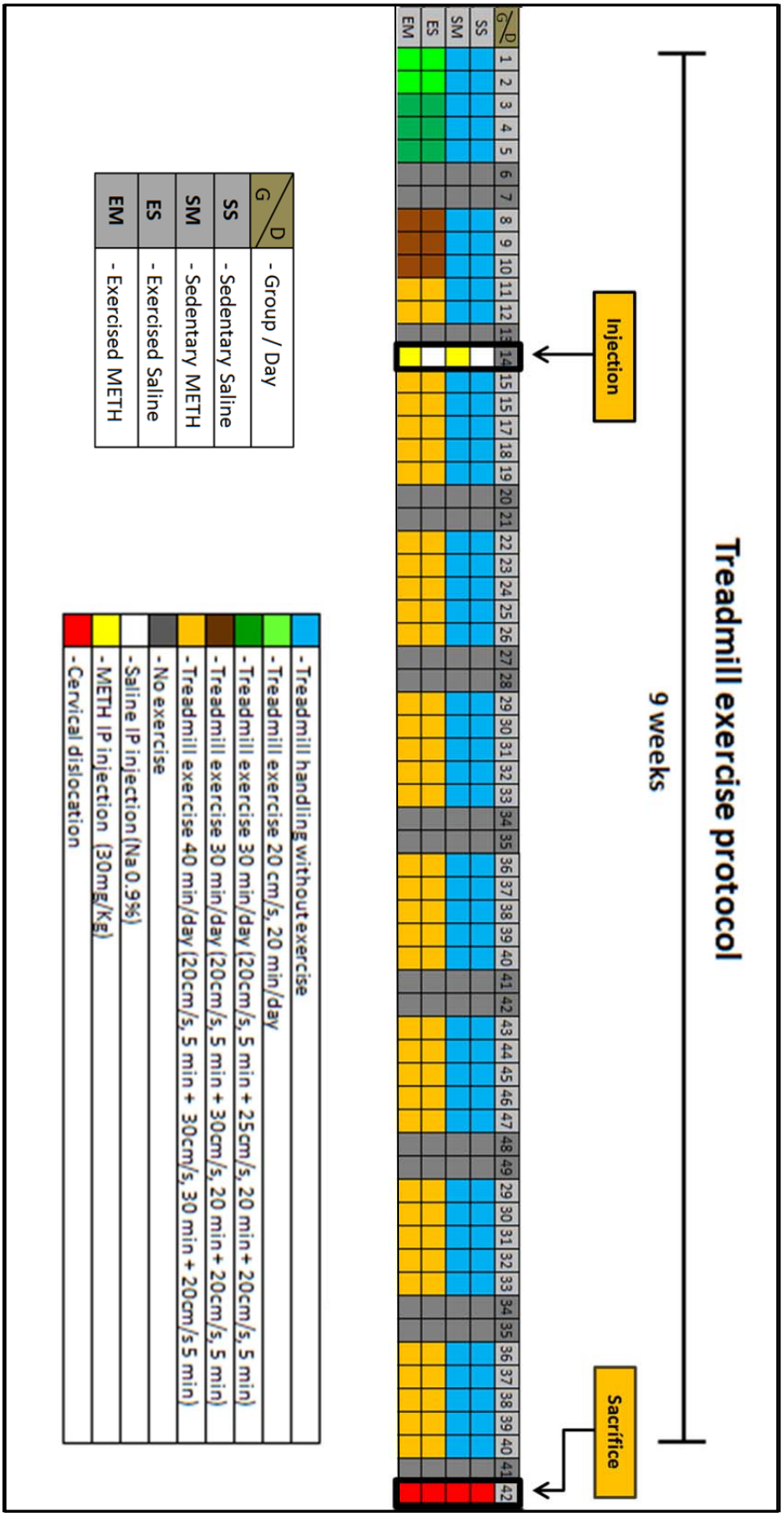

Figure 18. Mice treadmill exercise protocol established in our laboratory.

The exercised groups ran on the lane treadmill without receiving electric shocks. Exercise was performed at normal room temperature (RT), in a normal light setting, and with normal room noise. All animals were weight controlled once a week and the weight gain was comparable in the four groups (data not shown). The mice were sacrificed 48h after the exercise protocol by cervical dislocation.

3.4. Neurochemistry

METH produces a significant degree of striatal DA depletion and the damage to striatal terminals observed in METH abusers is indicative of METH toxic mechanisms (Thrash B et al., 2009). Therefore we used mice striata herein to assess dopaminergic injury METH-induced.

4.1. Monoamine assessment by HPLC-ECD

The striatal monoamine levels were assayed by HPLC-ECD as previously described (Kita et al., 1998; Fukumura et al., 1998; Imam and Ali, 2001; Pereira et al., 2006). The HPLC-ECD system used in our laboratory included a Gilson pump (model 307), a Gilson automatic injector (model 234) with a 50 µL loop, a Gilson detector (model 142) and the Software Unipoint v5.11 for data acquiring and analysis (Figure 19).

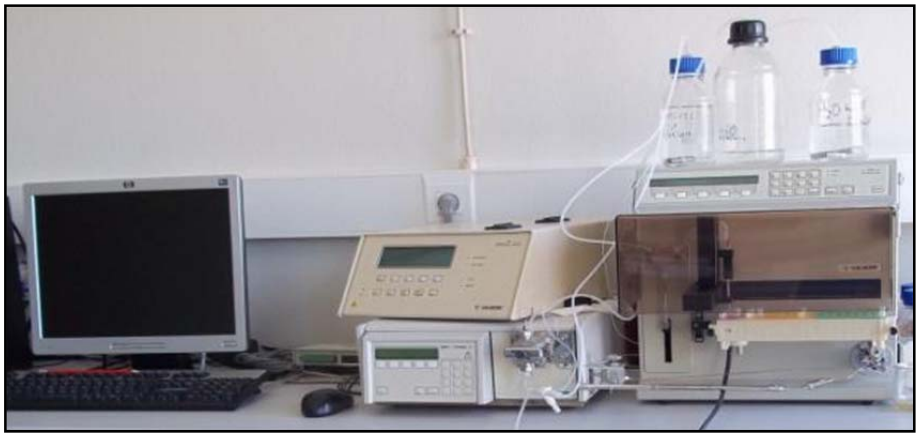

Figure 19. HPLC-ECD system used in the study for monoamine quantification. Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra.

Herein the tissues of the right striata were disrupted by ultrasonication in ice cold (3 pulses of 10s) in 250µl of 0.2 M perchloric acid (HClO4) and centrifuged (13000rpm, 7 min, 4°C). The supernatants were removed and filtered through a 0.2 µm Nylon microfilters (Spin-X® Centrifuge Tube Filter, Costar) at 10000rpm for 10 min at 4°C and stored at -80°C for further analysis. The pellets were neutralized and ressuspended in 250 µl 1 M NaOH, and total protein quantification was measured using a bicinchonic acid protein assay kit (Thermoscientific®) with bovine serum albumin (BSA) as standard. Samples of 50µL of supernatants were injected onto the HPLC-ECD system for separation and quantitation of DA, DOPAC and HVA. These compounds were separated on a reversed-phase Waters Spherisorb® ODS2 column (4.6 x 250 mm Analytical Cartridge; 5 µm) with a mobile phase designed to assess monoamines (Table 3). This mobile phase was degassed, filtered and pH was adjusted. The HPLC-ECD retention times for monoamines are shown in Table 4.

Table 2. Experimental conditions for HPLC-ECD

Mobile phase	
pH	4.5
Mobile phase	0.1 M sodium acetate trihydrate 0.1 M citric acid monohydrate 0.5 mM sodium octane sulphonate 0.15 mM EDTA 1 mM triethylamine 10 % methanol (v/v)
Flow rate	1.0 mL/min
Electrode detection	0.75 V
Sensitivity	2 nA/V

Table 3. HPLC-ECD - dopamine and metabolites retention times

Monoamine	**Retention time (min)**
DOPAC	6.64
DA	9.03
HVA	16.40

Dopamine and metabolites concentration was determined by comparison with peak areas of standards (Tables 5 and 6), and expressed in ng/mg of protein. Two calibration standards solutions at 50ng/ml were ran at the beginning at the end of each HPLC procedure each day as a quality control (Figures 20 and 21).

Table 4. Calibration standards that were ran at the beginning of HPLC-ECD procedure.

	Peak Width 1/2 HT	Peak Name	R. Time	Area
1	0.13	DOPAC	6.55	55879.55
2	0.18	DA	8.86	127995.89
3	0.31	HVA	15.93	47693.33
4	0.44	5-HT	24.58	149625.84

Image taken from Software Unipoint v5.11.

Table 5. Calibration standards that were ran at the end of HPLC-ECD procedure.

	Peak Width 1/2 HT	Peak Name	R. Time	Area
1	0.14	DOPAC	6.49	52560.00
2	0.18	DA	8.70	125559.15
3	0.30	HVA	15.88	52647.50
4	0.46	5-HT	23.98	140515.39

Image taken from Software Unipoint v5.11.

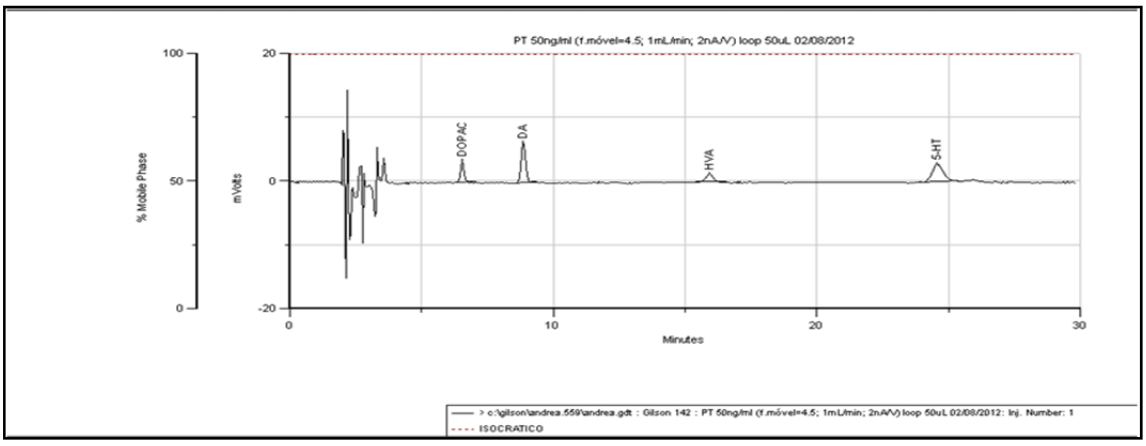

Figure 20. An illustrative chromatogram from calibration standards that were ran at the beginning of HPLC-ECD procedure. Image taken from Software Unipoint v5.11.

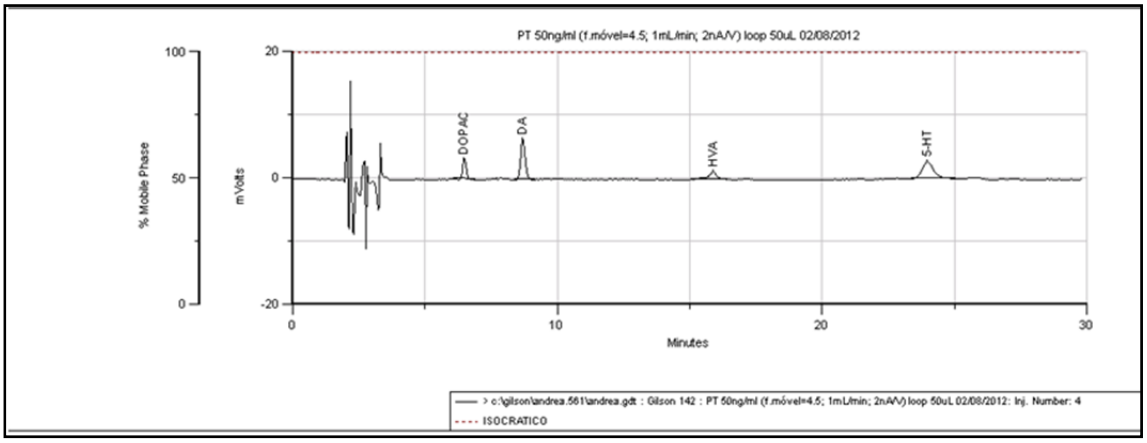

Figure 21. An illustrative chromatogram from calibration standards that were ran at the end of HPLC-ECD procedures. Image taken from Software Unipoint v5.11.

Standards for DA, DOPAC and HVA were purchased from Sigma-Aldrich. The other used chemicals (HPLC grade and pro analysis quality) were purchased from Sigma-Aldrich and Merck AG (Darmstadt, Germany).

4.2. Western Blot analysis

Western Blot technique (Figure 22) was used herein for measuring striatal proteins TH and GFAP levels according to Simões et al., (2008). This is an important technique used in cell and molecular biology to identify specific proteins from a complex mixture of proteins extracted from cells and tissues (Mahmood and Yang, 2012).

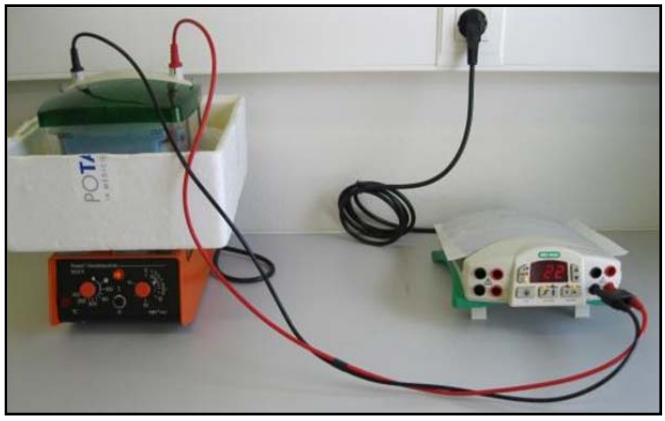

Figure 22. Western Blot system used in the study of protein expression. Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra.

Left striata were added to RIPA buffer lysis (NaCl 150mM; Tris-HCl 50 mM, pH=8; EGTA 5mM; 1% Triton X-100; 0.5% DOC; 0.1% SDS) supplemented with a protease inhibitor cocktail (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM dithiothreitol (DTT), 1µg/mL chymostatin, 1µg/mL leupeptin, 1µg/mL antipain, 5µg/mL pepstatin A - CLAP). The samples were disrupted by ice-cold ultrasonication (3 pulses of 15s) and centrifuged (13 000 rpm, 15 min at 4ºC), leading to a soluble supernatant fraction, corresponding to total extract and stored at -80°C for further analysis. The total protein concentration was measured using bicinchonic acid protein assay kit (Thermoscientific®).

Samples were denatured at at 98 ^{o}C for 5 minutes in denaturing solution 6x diluted (Tris-HCl, 0,5M, pH 6,8; SDS 10% (m/v); glycerol 30% (v/v), DTT 0,6M, bromophenol blue 0,01% (m/v)). Equal amounts of protein (3µg for TH and 10µg for GFAP) were loaded into the gels and separated by electrophoresis on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% gels. Then, proteins were transferred electrophoretically to polyvinylidene difluoride membranes (PVDF; Millipore, Madrid, Spain), and blocked with 5% non fat dry milk in phosphate-buffered saline solution (PBS, in mM: 137 NaCl, 2.7 KCl, 4.3 Na2HPO4, 1.47 KH2PO4; pH 7.4) containing 0,1% Tween-20 (PBS-T) for 1 h at RT. Blots were then incubated overnight at 4ºC with primary antibodies (Table 7) diluted in a solution of 5% skimmed milk (m/v) in PBS-T. Membranes were then incubated with alkaline phosphatase-conjugated IgG secondary (Table 7) prepared in PBS-T for 1h at RT. Finally, membranes were visualized on an imaging system (Thyphoon FLA 9000, GE Healthcare) using an enhanced chemifluorescence detection reagent (ECF, GE Healthcare). To confirm equal protein loading and sample transfer, membranes were reprobed with mouse anti-β-GAPDH 1:2000 (Abcam) or mouse anti-tubulin 1:5000 (Sigma-Aldrich). Densitometric analyses were performed using the Image Quant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA) and results were expressed as percentage of Sedentary Saline (SS) and presented as mean± standard error (SEM).

Table 6. Primary and secondary antibodies used for Western-blot analysis

Antibodies	Molecular weight (KDa)	Loading (µg)	Dilution	Reference	Company
Mouse anti-GFAP	50	10	1:1000	IF03L	Millipore
Mouse anti-TH	59-63	3	1:2000	MAB 318	Millipore
Mouse anti-GAPDH	40	-	1:2000	Ab 9484	Abcam
Mouse anti-β-Tubulin	55	-	1:5000	T 7816	Sigma-Aldrich
Goat anti-mouse	_	-	1:5000	A 3582	Sigma-Aldrich

3.5. Statistical analysis

The GraphPad Prism 5.0 software was used to analyze data. All values were expressed as mean ±S.E.M. The statistical significance for multiple comparisons was evaluated by two-way ANOVA (drug x exercise) and Bonferroni post-tests; the unpaired Student's t-test was used to compare two independent groups. Differences were considered significant at $P<0.05$.

CHAPTHER 4

Results

4.1. METH +/- Exercise: focusing on gross behavioral changes

Mice showed physical and behavioral changes following IP METH administration, including spiky hair (Figure 23), stereotypies and increased locomotor activity. All these changes stopped at the end of the day, and the treated mice presented a similar physical and behavioural phenotype to the saline groups.

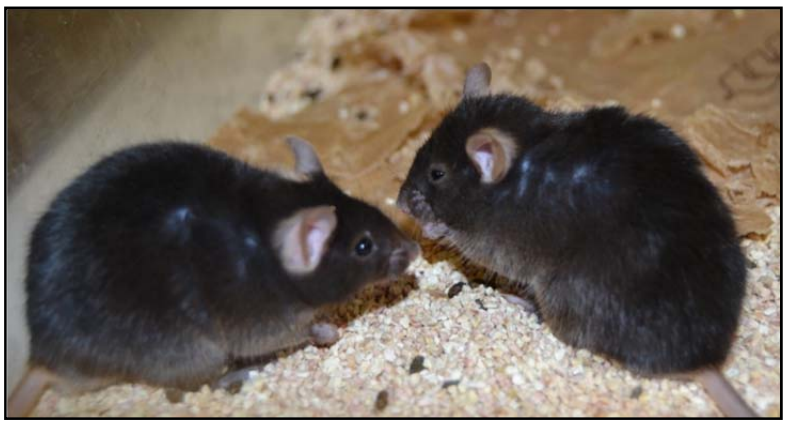

Figure 23. Mice post-METH injection. Animals presented stereotyped behavior and spiky hair. Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra.

No casualties were induced by METH. During the treadmill exercise the METH group did not show performance changes when compared with the Saline group.

4.2. Neurochemical analysis

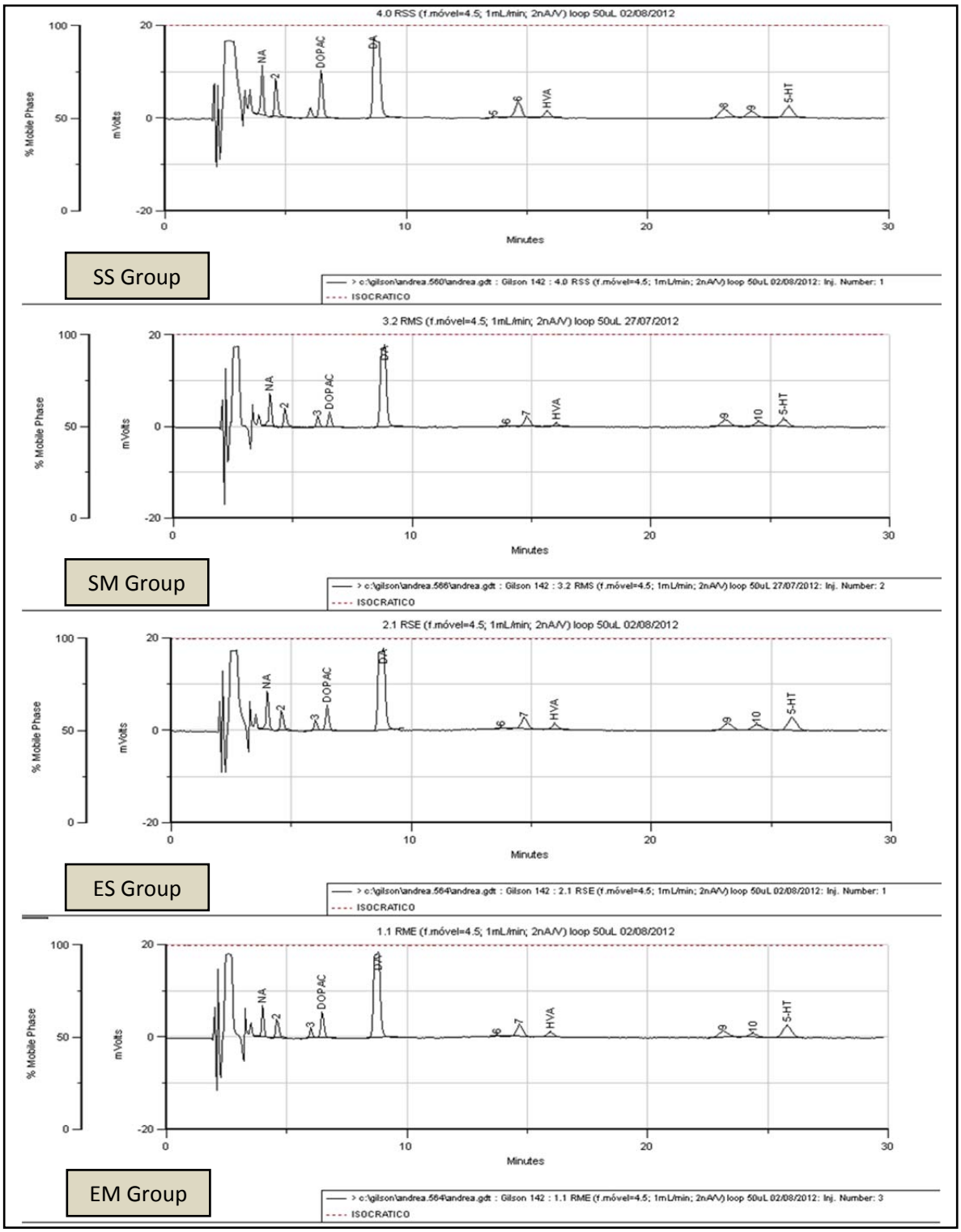

Figure 24. Four representative HPLC-ECD chromatograms from each experimental group. Image taken from Software Unipoint v5.11.

4.2.1. Striatal levels of DA

Figure 24 shows four representative chromatograms from each experimental group. A two-way ANOVA test disclosed that the Treatment x Exercise interaction was not significant in striatal DA levels (F=5.75; p=0.0276). However, METH induced DA depletion in the Sedentary group but not in the Exercised group (P<0.05). Exercise did not change striatal DA levels (P>0.05) (Figure 25).

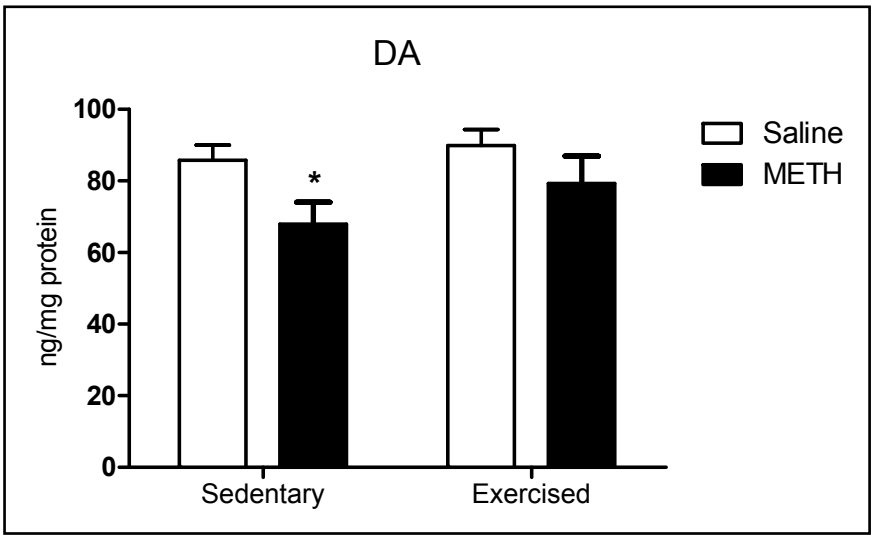

Figure 25. Effect of METH administration (30mg/Kg) and/or exercise on striatal DA levels (HPLC-EC). Data are presented as mean ± S.E.M. (n=6 per experimental group). * P < 0.05 versus SS group.

4.2.2. Striatal levels of DOPAC

A two-way ANOVA test disclosed that the Treatment x Exercise interaction was significant in striatal DOPAC levels (F=12.20; p=0.0023). METH induced DOPAC depletion in sedentary animals, while not in the exercised mice (*,#P<0.05). Exercise did not change striatal DOPAC levels (P>0.05) (Fig. 26).

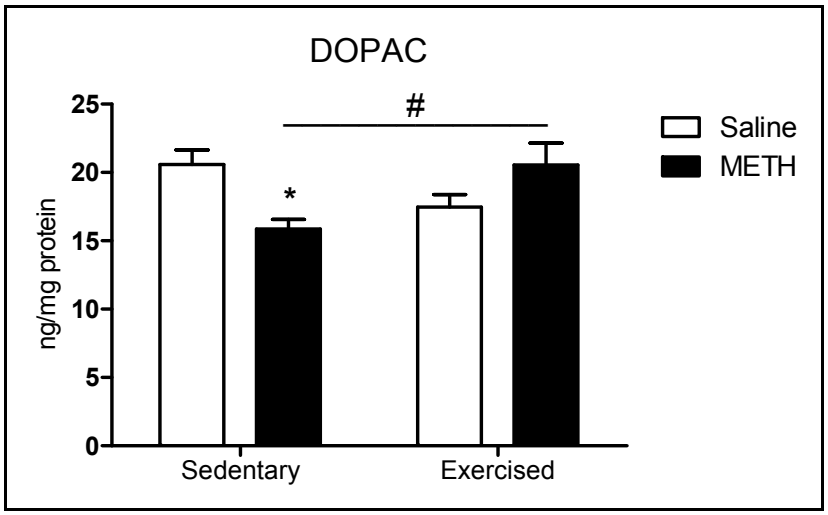

Figure 26. Effect of METH administration (30mg/Kg) and/or exercise on striatal DOPAC levels (HPLC-EC). Data are presented as mean ± S.E.M. (n=6 per experimental group). * P<0.05 versus SS group; #P<0.05 compared with each other.

4.2.3. Striatal levels of HVA

A two-way ANOVA test revealed that the Treatment x Exercise interaction was significant in striatal HVA levels (F=8.83; p=0.0078). METH induced HVA depletion in sedentary animals, while not in the exercised mice (*P<0.05 and #P<0.01). Exercise did not change striatal HVA levels (P>0.05) (Fig. 27).

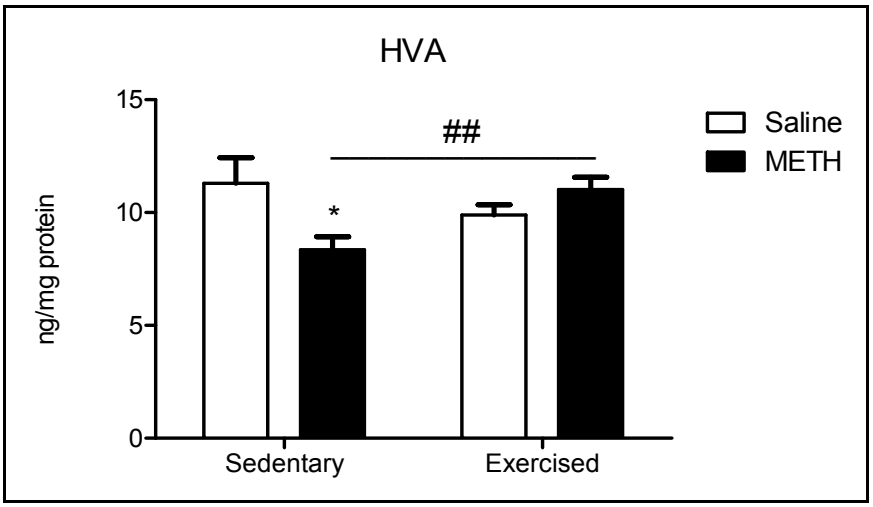

Figure 27. Effect of METH administration (30mg/Kg) and/or exercise on striatal HVA levels (HPLC-EC). Data are presented as mean ± S.E.M. (n=6 per experimental group). *P< 0.05 versus SS group; ##P< 0.01 compared with each other.

4.2.4. Striatal levels of TH

A two-way ANOVA test revealed that the Treatment x Exercise interaction was significant in striatal TH levels (F=12.06; p=0.0024). METH induced TH depletion in sedentary animals, while not in the exercised mice (*P<0.05 and #P<0.01). Exercise did not change striatal TH expression (P>0.05) (Fig. 28).

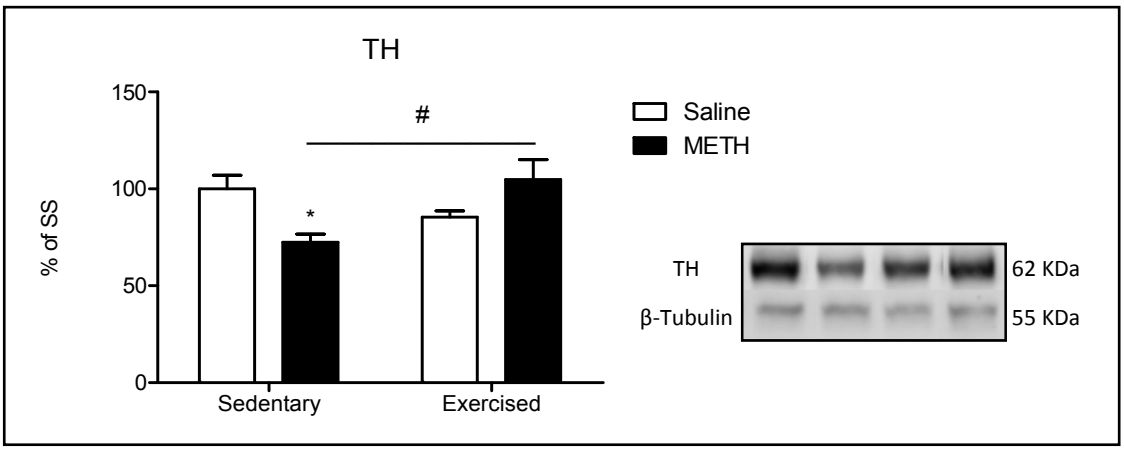

Figure 28. Effect of METH administration (30mg/Kg) and/or exercise on striatal TH levels (Western-blot). Data are presented as mean ± S.E.M. (n=6 per experimental group). *P<0.05 versus SS group; #P<0.05 compared with each other.

4.2.5. Striatal levels of GFAP

Neither METH nor exercise altered striatal GFAP expression (P>0.05). However, GFAP levels from EM group are significantly lower than those from both SM and ES groups (P<0.05) (Fig. 29).

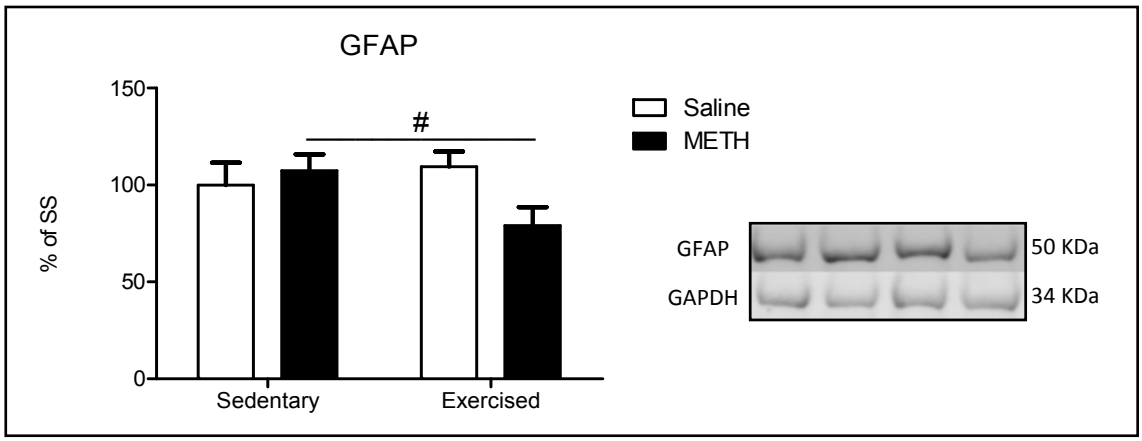

Figure 29. Effect of METH administration (30mg/Kg) and/or exercise on striatal GFAP levels (Western-blot). Data are presented as mean ± S.E.M. (n=6 per experimental group). *P<0.05 versus ES; #P<0.05, compared with each other.

CHAPTER 5

Discussion

Discussion

The purpose of this study was to investigate the impact of exercise on DAergic system and astrogliosis in the striata from mice exposed to METH and further explore possible mechanisms of exercise-induced neuroregeneration. We found that seven weeks after treatment METH imposed a significant decrease in DA and its metabolites and in TH in the Sedentary group when compared with the saline group ($P < 0.05$). This is clearly suggestive that METH induced a long lasting DAergic toxicity. These data are consistent with recent findings from our group (Silva et al. 2014). Additionally we and others provided robust evidence supporting that METH is highly toxic to striatal DAergic terminals (Pereira et al., 2002; Yamamoto and Bankson, 2005; Riddle et al., 2006; Krasnova and Cadet, 2009; Gouzoulis-Mayfrank and Daumann, 2009; Yamamoto et al., 2010) at early and late time-points following METH administration.

We newly found that a 7 week-protocol treadmill exercise recovered striatal DAergic homeostasis following a single-high METH dose. This is highly suggestive that running exercise has neuroregenerative properties in DAergic systems. Treadmill exercise was proven to have beneficial effects in nigrostriatal DAergic pathway in different experimental settings. For example, Park et al. (2013) demonstrated that this type of exercise ameliorated nigro-striatal DAergic neuronal loss, resulting in the improvement of spatial learning ability after neonatal hypoxic ischemia brain injury. Another study clearly showed that treadmill exercise enhanced the survival of DAergic neurons in the substantia nigra and also their fibers projecting into the striatum in 6-hydroxydopamine-induced Parkinson's rats (Yoon et al., 2007). These authors argued that treadmill exercise may provide therapeutic value for the treatment of Parkinson's disease patients. More recently, Smith et al. (2011) showed that treadmill exercise improved gait performance and increased physical activity while promoting increased protein expression of striatal TH in mice injected with the DAergic toxin MPTP. Importantly, our treadmill data in mice is consistent with running wheel data in rats reported by O´Dell et al. (2012). These authors showed that when rats engaged in voluntary aerobic exercise for 3 weeks before and 3

weeks after a binge regimen of METH, exercise significantly ameliorated METH-induced decreases in striatal TH. However, O´Dell and Marshall (2014) showed that prior exercise provided no protection against METH-induced damage to striatal DA terminals. Our results in combination with O´Dell´s suggest that running exercise is endowed with neuroregenerative but not with neuroprotective properties in a setting of METH-damaged terminals.

Altough METH-induced damage to DAergic terminals is long-lasting, it is not permanent. In fact, DAergic markers returned to control levels over the course of about 1 year (Cass and Manning, 1999). Therefore, it is likely that running exercise both accelerated and facilitated the compensation/repair processes set in motion after METH injury, as suggested by O´Dell and collaborators (2012, 2014). A possible mechanism by which exercise might cause accelerated recovery is the induction of growth or neurotrophic factors including glial derived neurotrophic factor (GDNF). For example, previous studies have shown that treadmill exercise increased GNDF levels in the DA-denervated striatum of rats after unilateral 6-OHDA injections (Cohen et al., 2003; Smith and Zigmond, 2003). Increased neurotrophic factors might foster plasticity and repair in damaged DAergic terminals as suggested by O´Dell et al. (2012). Finally, Kleim et al. (2003) suggested that motor exercise may prime the brain to respond more adaptively to injury, in part by upregulating trophic factors such as GDNF and others (fibroblast growth factor-2 - FGF-2 or brain-derived neurotrophic factor - BDNF). The effects of exercise on striatal DA neurotransmission are unclear in normal settings (Wang et al., 2000). Herein we show that treadmill exercise did not change striatal DAergic markers. This is consistent with Fisher et al. (2004) findings showing that striatal TH levels from exercised mice were not significantly different from sedentary mice. However, Smith et al. (2011) showed that treadmill exercise lead to an increase in striatal TH levels. This apparent discrepancy might stem from different treadmill exercise protocols.

Astrocytes play a major role in METH-induced neurotoxicity (Krasnova and Cadet, 2009). METH did not change striatal GFAP levels in the sedentary group, 7 weeks post-METH. This is consistent with the transitory striatal reactive astrogliosis upon METH administration in mice (Silva et al., 2014). There is scarce information on the impact of physical activity on astrocytes. Herein we show that treadmill exercise did not change GFAP levels. This is consistent with findings in rat from Dutra et al. (2012). However, it

was previously shown that exercise induced an increase in striatal astrocyte density (Li et al., 2005). These dissonant observations warrant for further clarification of this matter. GFAP levels in the EM group were significantly lower compared to the SM and ES groups ($P < 0.05$). Although unexpected, this observation further confirms that the exercise has a beneficial impact in the striatum from METH-injected animals. Moreover, these data might suggest that there is a reduction in the expansion of astrocytes to accommodate an increase in synaptic function triggered by exercise in the METH-group. This hypothesis warrants further scrutiny.

CHAPTER 6

Conclusion

Conclusion

This study is suggestive that exercise is provided with regenerative properties to DAergic striatal terminals in a setting of METH-induced DAergic terminal toxicity. However, the mechanism responsible for this positive effect warrants further scrutiny. This work adds to the suggestion that treadmill exercise should be included in a treatment plan for METH addicts.

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