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# Treadmill exercise exerts an antidepressant-like effect to mice submitted to a neurotoxic methamphetamine dose

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Frederico Pereira (Universidade de Coimbra) e do Professor Doutor Rui de Carvalho (Universidade de Coimbra)

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# Abbreviations

**5-HIAA** – Hydroxyindoleacetic acid

**5-HT** – 5-hydroxytryptamine; serotonin

**5-HTP** – 5-hyfroxytryptophan

**AADC** – L-amino acid decarboxylase

**AADC** – L-aromatic amino acid decarboxylase

**ACTH** – Adrenocorticotropic hormone

**ADHD** – Attention deficit hyperactivity disorder

**AMPH** – Amphetamine

**ATS** – Amphetamine-type stimulants

**BBB** – Blood brain barrier

**BDNF** – Brain-derived neurotrophic factor

**BH4** – Tetrahydrobiopterin

**CNS** – Central nervous system

**Cont** – Control

**CREB** – cAMP response element-binding protein

**CRF** – Corticotropin-releasing factor

**DA** – Dopamine

**DAT** – Dopamine transporter

**DOPAC** – 3,4 – dihydroxyphenylacetic acid

**ENS** – Enteric nervous system

**FST** – Forced-swim test

**GDNF** – Glial cell–derived neurotrophic factor

**HPA** – Hypothalamic-pituitary-adrenal

**HPLC** – High pressure liquid chromatography

**HVA** – Homovanillic acid

**IBS –** Irritable bowel syndrome

**L-TRP**- L-tryptophan

**MAO** – Monoamine oxidase

**MAO-A** – Monoamine oxidase A

**MAOIs** – Monoamine oxidase inhibitors

**MDD** – Major depressive disorder

**MDD** – Major depressive disorder

**MDMA** – 3-4-methylenedioxymethamphetamine

**METH** – Methamphetamine

**NA** – Norepinephrine

**Nac** – Nucleus accumbens

**OF** – Open-field test

**PFC** – Prefrontal cortex

**PNS** – Peripheral nervous system

**SAL** – Saline

**SED** – Sedentary

**SERT** – Serotonin transporter

**SNS** – Sympathetic nervous system

**SSRIs** – Serotonin-specific reuptake inhibitor

**TCA** – Tricyclic antidepressants

**TH** – Tyrosine hydroxylase

**TPH** – Tryptophan hydroxylase

**$TPH_2$** – Tryptophan hydroxylase 2

**UNODC** – United Nations Office on Drugs and Crime

**$VMAT_2$** – Vesicular monoamine transporter 2

**VTA** – Ventral tegmental area

**WHO** – World Health Organization

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# Resumo

Demonstrámos recentemente, que uma dose única e elevada de metanfetamina (METH) causou um fenótipo depressivo de longa duração, aferido pelo aumento do tempo de imobilidade no teste de *tail suspension*. Tem sido demonstrado que o exercício físico alivia os sintomas depressivos.

Propomo-nos confirmar e caracterizar mais detalhadamente o fenótipo depressivo causado por uma única injecção neurotóxica de METH e investigar a correlação entre a homeostase de 5-HT no córtex frontal e hipocampo e o efeito antidepressivo de exercício físico.

Murganhos adultos C57BL/6 foram submetidos a um programa de exercício em *treadmill* (cinco dias por semana durante sete semanas) a partir de 24 h após uma única dose elevada de METH (30 mg/kg, i.p.), tendo sido organizados da seguinte forma (n=8/grupo): salino/sedentário; METH/sedentário; salino/exercício e METH/exercício. Em seguida, avaliou-se a exploração/actividade locomotora, anedonia e desespero (comportamentos do tipo depressivo) numa fase tardia da privação de METH (49 dias) através dos testes *open-field*, *splash* e *forced-swim*, respectivamente. Os teores totais de 5-HT no cortex-frontal e hipocampo (49 dias pós-METH) foram avaliados por cromatografia líquida de fase reversa e de alta eficiência (HPLC). Avaliámos o impacto inicial (3 dias pós-METH) de METH nos níveis totais de 5-HT do hipocampo noutro grupo de murganhos (16).

É importante registar que a METH diminuiu o tempo de *grooming* dos murganhos sedentários, no teste *splash*. Isto demonstra que esta dose de METH induz um comportamento do tipo anedónico. Por outro lado, confirmou-se ainda que a METH induz um comportamento do tipo depressivo de longa duração. Além disso, o exercício em *treadmill* reverteu o tempo de *grooming* no teste *splash*, 7 semanas após a injecção de METH. Este efeito antidepressivo foi ainda aferido por uma diminuição do tempo de imobilidade e um aumento do tempo de natação no teste *forced-swim* exibido pelos murganhos, control ou submetidos à METH. Adicionalmente, o exercício aumentou a actividade locomotora, o comportamento exploratório e a razão distância central - distância total nos murganhos control ou submetidos à METH no teste *open-field*. Isto é consistente com o facto de o exercício físico induzir um estado de bem-estar emocional, independentemente do tratamento farmacológico. Por outro lado, a METH não alterou significativamente os parâmetros motores de murganhos sedentários. Isto é relevante, visto

que sugere que a actividade locomotora não contribuiu para o comportamento do tipo anedónico observado.

A análise neuroquímica mostrou que a METH provocou uma deplecção robusta de 5-HT no hipocampo nos primeiros 3 dias após a injecção de METH, que foi recuperada 49 dias após o tratamento. Além disso, o exercício físico não teve um impacto significativo nos níves hipocampais de 5-HT. Adicionalmente, nem o exercício físico nem a METH alteraram significativamente os teores toatis de 5-HT.

É importante destacar, que demostrámos que um programa de sete semanas de exercício em *treadmill* promoveu um efeito antidepressivo em murganhos intoxicados com METH. No entanto, este benefício do exercício não está associado a altenações na homeostase serotonérgica fronto-cortical/hipocampal.

**Palavras-chave:** metanfetamina, neurotoxicidade, depressão, serotonina, exercício físico, córtex frontal, hipocampo, murganho.

# Abstract

Recently we demonstrated that a single high neurotoxic dose of methamphetamine (METH) caused a long-lasting depressive phenotype as gauged by increased immobility time in the tail suspension test. It has been shown that physical exercise alleviates depressive symptoms.

This study aims to further characterize the long-term depressive phenotype caused by a single neurotoxic METH-injection and to probe for a correlation between frontal-cortical and hippocampal 5-HT homeostasis and the antidepressant effect of physical exercise.

Adult C57BL/6 mice were submitted to a treadmill exercise program (five days a week for seven weeks) starting 24 h post-single high dose of METH (30 mg/kg, i.p.) and were organized as follows (n=8/group): saline/sedentary; METH/sedentary; saline/exercise and METH/exercise. Then, we assessed the exploration/locomotor activity, anhedonia and despair (depressive-like behaviours) at a late stage of METH withdrawal (49 days) by open-field, splash and forced-swim tests, respectively. Frontal cortical and hippocampal (49 days post-METH) levels of 5-HT were evaluated by reversed-phase high performance liquid chromatography (HPLC). Another set of mice (16) was used to evaluate early impact (3 days post-METH) of METH on 5-HT hippocampal levels.

Remarkably METH decreased the time that sedentary mice spent grooming. This shows that this METH dose induces anhedonia-like behavior. This further confirms that METH induces a long-lasting depressive-like behaviour. Remakably treadmill exercise reversed the time mice spent grooming in the splash test 7 weeks post-injection. This antidepressant-like effect was further gauged by a decreased immobility time and increased swimming time in the forced swimming test exhibited by both saline- and METH-exercised animals. Additionally, exercise increased locomotor activity, exploratory behavior and the central distance – total distance ratio in both saline and METH treated mice in the open field. This is consistent with physical exercise inducing a high-mood state, irrespectively of drug treatment. On the other hand, METH did not alter significantly the motor parameters from sedentary mice. This is relevant since locomotor activity did not contribute to the anhedonic-like behavior seen herein. The neurochemical analysis showed that METH triggered a robust 5-HT hippocampal depletion as early as 3 days post-METH, which recovered 49 days post-tretament. Additionally, physical exercise failed to have any

significant impact on hippocampal 5-HT levels. Moreover, neither METH nor physical exercise altered frontal cortical 5-HT levels.

Importantly, we present new evidence that a 7 weeks treadmill program provided an antidepressant effect to METH-intoxicated mice. This exercise benefit is not associated with changes in the frontal cortical/hippocampal serotonergic homeostasis.

**Keywords:** methamphetamine, neurotoxicity, depression, serotonin, physical exercise, frontal cortex, hippocampus, mice.

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# CHAPTER 1

# INTRODUCTION AND AIMS

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# 1. Drug Addiction

Drug addiction is a major health and social issue due to high rates of morbidity and mortality that are accompanied by bouts of violence and legal problems, thus imposing burdening individual and societal costs (Al-Haggar, 2014). This medical and societal problem is considered to be a chronic, relapsing brain disorder. Particulalrly, brain addiction is envisaged as a malfunction of the brain's hedonic tone regulation and the motivational system (Müller & Homberg, 2014). This psychiatric disorder is characterized by compulsive drug seeking, by their continued use despite serious negative socioeconomic and health consequences and marked withdrawal symptoms (Cami & Farré, 2003; National Institute on Drug Abuse (NIDA, 2012); Koob & Volkow, 2010 and McAvoy, 2009).

Collapsing the cycles of impulsivity and compulsivity yields a composite addiction cycle composed of three stages-binge/intoxication, withdrawal/negative affect, preoccupation/anticipation-in which impulsivity often dominates at the early stages and impulsivity combined with compulsivity dominates at the later stages (Koob & Volkow, 2010). As an individual moves from impulsivity to compulsivity, a shift occurs from positive reinforcement driving the motivated behavior to negative reinforcement and automaticity driving the motivated behavior. These three stages are conceptualized as interacting with each other, becoming more intense, and ultimately leading to the pathological state known as addiction (Figure 1).

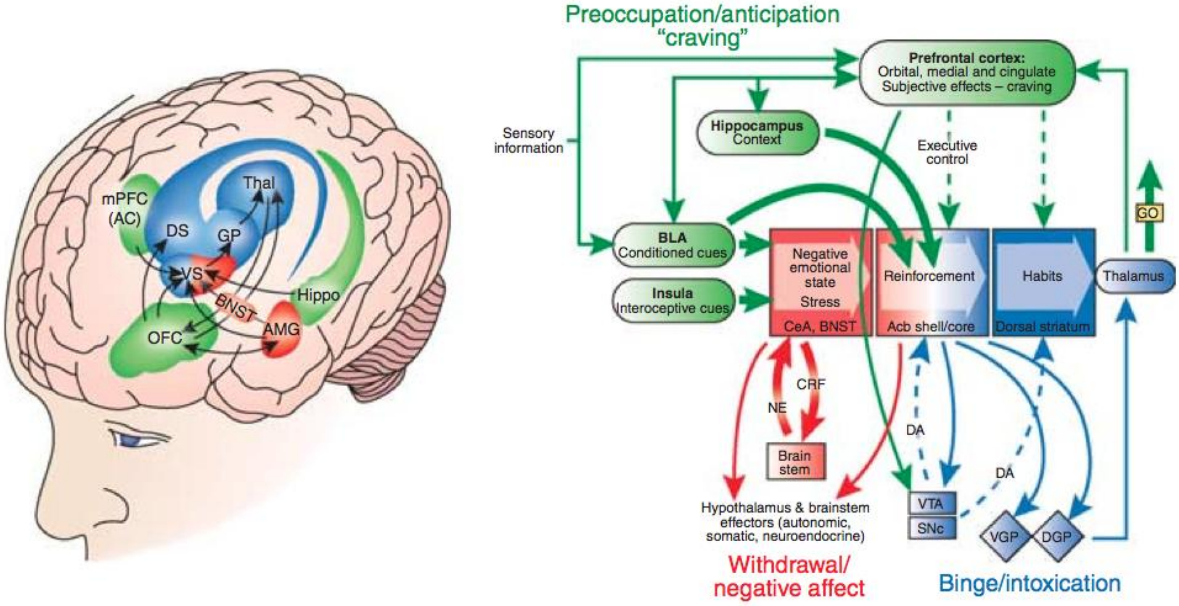

**Figure 1** – Neurocircuitry schematic illustrating the combination of neuroadaptations in the brain circuitry for the three stages of the addiction cycle that promote drug-seeking behavior in the addicted state. Note the activation of the ventral striatum/dorsal striatum/extended amygdala driven by cues through the hippocampus and basolateral amygdala and stress through the insula. The frontal cortex system is compromised, producing deficits in executive function and contributing to the incentive salience of drugs compared to natural reinforcers. Dopamine systems are compromised, and brain stress systems such as CRF are activated to reset further the salience of drugs and drug-related stimuli in the context of an aversive dysphoric state (taken from Koob & Volkow, 2010).

The binge–intoxication stage of the addiction cycle dependes upon the reinforcing effects of drugs that may engage reward neurotransmitters (including dopamine and opioid peptides) and associative mechanisms in the nucleus accumbens shell and core and then engage stimulus–response habits that depend on the dorsal striatum (Koob & Volkow 2010).

The preoccupation–anticipation (craving) stage involves key glutamatergic projections to the extended amygdala and nucleus accumbens from the prefrontal cortex (for drug-induced reinstatement of drug seeking) and from the basolateral amygdala (for cue-induced reinstatement of drug seeking) (Kalivas et al., 2003). Compulsive drug-seeking behaviour is thought to engage ventral striatal–ventral pallidal–thalamic–cortical loops that could subsequently engage dorsal striatal–dorsal pallidal–thalamic–cortical loops (Vanderschuren et al., 2005), both of which are exaggerated by concomitant decreased activity in reward circuits (Koob et al., 2005, 2008).

The neural substrates and neuropharmacological mechanisms for the negative motivational effects of the withdrawal–negative affect stage of the addiction cycle may involve not only disruption of the neural systems implicated in the positive reinforcing effects of drugs, but also recruitment of brain stress systems (Koob et al., 2008).

Importantly, a new functional model also suggests that specific adaptations in the 5-HT system render the nervous system susceptible to the transition to compulsive drug use behaviours (Müller & Homberg, 2014).

## 1.1 Amphetamine-type stimulants

Amphetamine (1-methyl-2-phenethylamine) (AMPH), which is the first member of a group of compounds that have similar structures and biological properties and are collectively called “amphetamine-type stimulants” (ATS), was first synthesized by a Romanian chemist named Lazar Edeleanu at the University of Berlin in 1887. However, it

was not used clinically until Gordon A. Alles re-synthesized the drug in the 1920s for use in medical settings as a bronchodilator to treat asthma and colds (Benzedrine©) (Menhard, 2006; Berman et al., 2008; Cunha-Oliveira et al., 2013).

The most popular ATS include methamphetamine - synthesized six years later - 3-4-methylenedioxymethamphetamine (MDMA) and methylphenidate (Ritalin©, Concerta©, Methylin©), patented in 1914 and in 1954, respectively (McDowel & Kleber, 1994; Morton & Stockon, 2000, McCornack & Buckley, 2006). While methamphetamine is more potent than the parent compound, amphetamine has moderate hallucinogenics properties (Cunha-Oliveira et al, 2013).

ATS are indirect sympathomimetic and act in the nervous sytem by blocking transporter-mediated reuptake of biogenic amines dopamine (DA), serotonin (5-HT) and noradrenaline (NA) and by triggering an aberrant release of these amines from the presynaptic terminal into the synaptic cleft through the reversal of the amine transporters (Sulzer et al., 2005). Therefore, ATS produce a series of effects in mind and body including increasing energy, heart rate, respiration, and mental alertness, elevating mood and self-confidence, decreasing fatigue and sleep, producing euphoria and appetite suppression (Cunha-Oliveira et al., 2013; Berman et al., 2008).

While amphetamine, METH and methylphenidate have long been used for treating various disorders such as attention deficit hyperactivity disorder (ADHD), narcolepsy, and obesity, they also may cause addition, profound effects on mental function and behavior, and can produce neurodegeneration (Sulzer et al., 2005; National Institute on Drug Abuse (NIDA; 2012); Koob & Volkow, 2010 and McAvoy, 2009).

## 1.2 Epidemiology of abuse amphetamine-type stimulants

According to the World Drug Report 2012, which was issued by UNODC, amphetamines (amphetamine and methamphetamine) are the prescription drugs most commonly abused by adolescents and young adults, and illicit amphetamines abuse ranks second place in young adults, with an estimated prevalence of 0.3-1.2% in 2010 (between 14.3 million and 52.5 million consumers) (Table 1; Berman et al., 2008).

**Table 1 –** Drug Consumption in 2010, (UNODC). Information taken from World Drug Report (2012).

| **Annual prevalence and number of illicit drug users at the global level, 2010** | **Prevalence (percentage)** | | **Number (thousands)** | |
|---|---|---|---|---|
| | Low | High | Low | High |
| Cannabis | 2.6 | 5.0 | 119 420 | 224 490 |
| Opioids | 0.6 | 0.8 | 26 380 | 36 120 |
| Opiates | 0.3 | 0.5 | 12 980 | 20 990 |
| Cocaine | 0.3 | 0.4 | 13 200 | 19 510 |
| Amphetamine-type stimulants | 0.3 | 1.2 | 14 340 | 52 540 |
| "Ecstasy" | 0.2 | 0.6 | 10 480 | 28 120 |
| Any illicit drug | 3.4 | 6.6 | 153 000 | 300 000 |

In 2011, between 3.6 and 6.9 per cent of the adult population were estimated to have used an illicit substance in the preceding year. The prevalence of illicit drug use and the numbers of drug users with drug use disorders or dependence have remained stable (World Drug Report, 2013). While the prevalence of use of cocaine, amphetamines and "ecstasy"- group substances appears to have followed a declining trend between 2009 and 2011, the prevalence of cannabis, opioids, and opiates use has risen up (Figure 2). Nevertheless, since 2008 there has been an overall 18 per cent increase in the estimated total number of people who had used an illicit substance in the preceding year, which to some extent reflects both an increase in the global population and a slight increase in the prevalence of illicit drug use.

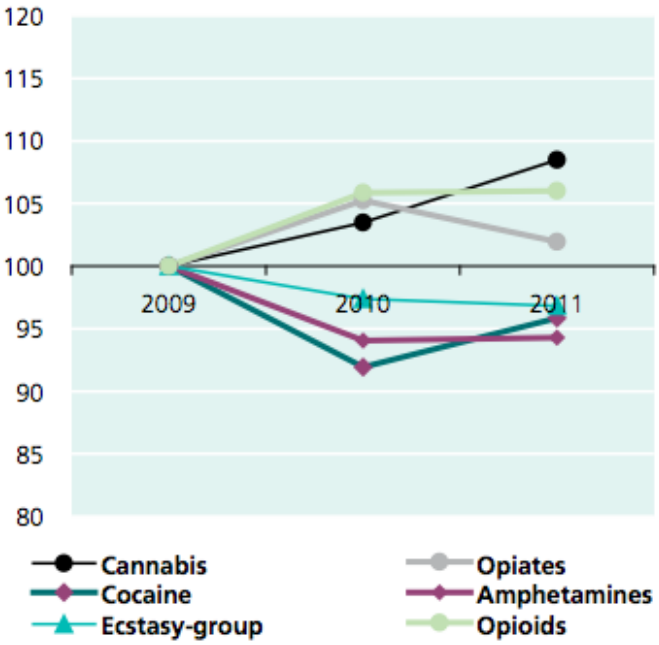


**Figure 2 –** Trends in the prevalence of different drugs, 2009-2011. Information taken from World Drug Report (2013).

According to World Drug Report (2013), Oceania, North America and Central America represent the regions with the highest prevalence of abuse of amphetamines. Moreover Southeast and Central Asia have been witnessing a growth in its abuse in recent years (Figure 3).

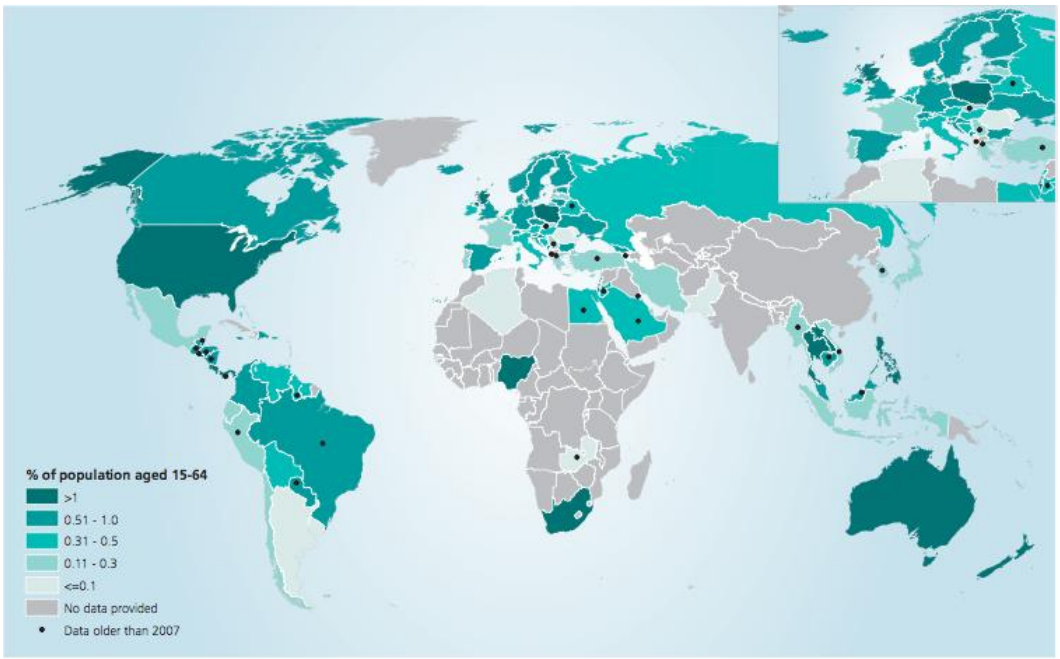


**Figure 3** – Use of ATS in 2011 (or latest year available). Information taken from World Drug Report (2013).

According to the National Survey on the Use of Psychoactive Substances in the General Population, Portugal 2012, amphetamines use prevalence increased 0.4% from 2001 to 2007 and youngest population is increasing its use in Portugal. The Algarve region, followed by Lisbon and Tagus Valley, has the highest rates of amphetamines use in both consumption throughout life and in continuation rates (Amaral & Guimarães 2012).

Relatively to METH, nearly 25 million people worldwide are estimated to have used methamphetamine (Buxton & Dove, 2008; McAvoy, 2009), a total that exceeds the number of people who abuse heroin and cocaine and makes METH the second most widely abused drug after cannabis. This is consistent with METH having become very popular due to its inexpensive production, low cost of acquisition, and durability in terms of effects (Krasnova & Cadet, 2009; Koob & Volkow, 2010), becoming a global epidemic (Barr et al., 2006).

## 1.3 Methamphetamine and Serotonin

### 1.3.1. Methamphetamine: pharmacokinetics and pharmacodynamics

Methamphetamine was introduced in the 1930s as a bronchodilator for the treatment of nasal and bronchial congestion associated with colds (Meng et al., 1999; Guerreiro & Carmo et al., 2011), and was originally synthesized from ephedrine by the Japanese pharmacologist Nagayoshy Nagai in 1893, but it was not widely used until World War II when Japan, Germany, and the United States gave it to military personnel to combat fatigue and increase endurance and performance (Freese et al., 2002; Meredith et al., 2005). METH can also be synthesized through pseudoephedrine reduction or a condensation of phenylacetone and methylamine (Cho, 1990, 2001). The produced METH is a lipid-soluble pure base form, which is volatile and evaporates if left exposed to room air. The producer, therefore, tipically uses hydrochloride to convert it to methamphetamine-HCL powder, which is water-soluble. This METH salt is marketed on the streets as “speed”, “crank”, “go”, “crystal” or methamphetamine (Derlet, 1990; McAvoy, 2009).

The additional methyl group found in METH confers higher lipid solubility to this molecule, comparatively to amphetamine, which results in greater blood brain barrier (BBB) permeability (Aron & Paulus, 2007), enhanced stability against enzymatic degradation by monoamine oxidase (MAO), and hence longer duration of action (Barr et al., 2006) (Figure 4).

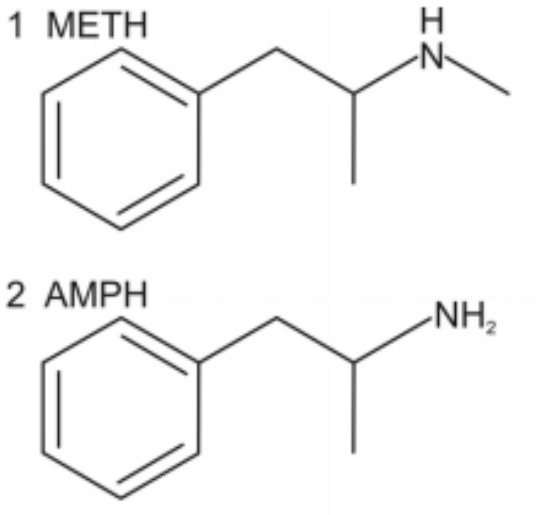


**Figure 4 –** Chemical structure of methamphetamine (METH) (1), as well as the closely related psychostimulant d-amphetamine (AMPH) (2). Adapted from Barr et al., 2006.

The clinical toxicology of drugs of abuse depends on the administration pathway, which affects its bioavailability (affecting the onset and extent of the psychotropic effects), the biodistribution (and therefore the exposure of target organs), and biotransformation or metabolism, which occurs mainly in the liver (affecting the nature and concentration of toxic compounds in the organism). The intensity and onset of a drug's effects are determined by the rapidity of its delivery to the central nervous system (CNS). Drug users learn to optimize the delivery of the drug to the brain and to maximize the bioavailability of the drug by adapting the methods and routes of administration. (Cunha-Oliveira et al., 2013).

In addition to the traditional routes of administration, such as oral ingestion, intravenous injection, and nasal snorting, METH can be smoked and also dissolved sublingually, or solubilized and consumed in a beverage (Meng et al., 1999). When administered intravenously or smoked, small doses of METH have prominent central stimulant effects, acting almost immediately and causing intense pleasure (rush or flash) that last only a few minutes (Schepers, 2003; Kish, 2008). Thus, these two pathways consumption allow greater drug concentration at the sites of action at the level of the CNS and, therefore, provide greater potential for addiction and increase the risk of overdose (McAvoy, 2009). When consumed intranasally or orally, METH effect is neither so immediate nor so intense when compared to intravenous and inhalation routes, because of lower bioavailability of the former routes. After oral ingestion, METH is rapidly absorbed, due to being highly lipid soluble, with peak plasma levels occurring within 2.6–3.6 h

(Cunha-Oliveira et al., 2013). Overall, effects of METH typically persist for 4-8h, but the residual effects can last up to 12h (McAvoy, 2009). A typical daily dose of oral METH for the treatment of ADHD in children is 20-25 mg (Kish, 2008). However, the required dose to produce a euphoric effect, typical of this drug, is 40 to 60 mg/day. Common abused doses of METH are 100-1000 mg daily, and up to 5000 mg in chronic binge use (McAvoy, 2009).

The desired acute effects include well-being, increased alertness, increased activity, excitement, and decreased appetite and anxiety. METH intoxication initially produces excessive stimulation of the sympathetic nervous system, resulting in marked tachycardia, hypertension, pupillary dilation, diaphoresis, tachypnea, peripheral hyperthermia and hyperpyrexia (Meredith et al., 2005; Homer et al., 2008). These effects can last for several hours because the elimination half-line of METH ranges from 10 to 12h (Schepers et al., 2003).

High doses may result in restlessness and agitation, and excessive doses may produce stereotypic behaviors (repetitive and automatic acts). Medical problems associated with excessive dosages include cerebral hemorrhage, stroke, seizure, hyperthermia, arrhythmias, coma, and death (NIDA, 1998). Chronic METH addicts often present altered brain structure and function underlying deficits in attention, working memory and decision-making and altered behavioral and cognitive functions (Krasnova & Cadet, 2009).

### 1.3.2. Serotonin Biosynthesis

Serotonin (5-hydroxytryptamine, 5-HT) is one of the ubiquitous molecules acting as messengers, well known as a neurotransmitter and neuromodulator (Berumen et al., 2011). 5-HT is phylogentically ancient and evolved prior to the apperanece of neurons (Muller & Jacobs, 2010). This biogenic amine was first isolated from mammalian organism in 1946 and then from the brain 7 years later (Hoyer et al., 2001; Hannon & Hoyer, 2008) and was named by Rapport et al. (1948).

The molecular machinery governing serotonin biosynthesis across serotonergic neurons is well characterized and also well conserved among vertebrates and invertebrates (Curran & Chalasani, 2012).

Figure 5 depicts synthesis and metabolism of 5-HT. This is a monoamine having an indoleamine group (Nishida, 2007) and being synthesized from L-tryptophan. This precursor is converted into L-5-hydroxytryptophan (L-5-HTP) by an enzymatic reaction

catalyzed by tryptophan hydroxylase-2 ($TPH_2$). Then, the enzyme aromatic L-amino acid decarboxylase (AADC) converts L-5-HTP to 5-HT. Subsequently, vesicle monoamine transporters (type 2) ($VMAT_2$) are responsible for packaging serotonin into vesicles. Evidence suggests that stored 5-HT is released in an even sprinkler-type fashion termed volume transmission, and functional concentrations of this neurotransmitter are maintained several microns from release sites (Doubert & Condron, 2010). Extracellular 5-HT binds to serotonin receptors on pre-and post-synaptic sites.

The signal is terminated when unbound serotonin is taken back into the presynaptic cells by reuptake transporters (SERT), thereby limiting 5-HT extracellular concentration. Finally, the enzymatic degradation of brain 5-HT is mainly mediated by monoamine oxidase MAO-A and, in the absence of this enzyme, by its cognate isoenzyme MAO-B. (Bortolato er al., 2008). In addition, since MAO-B is the only isoenzyme expressed in platelets (Bond & Cundall, 1977), it may play an important function in the regulation of plasma 5-HT levels. This metabolic step origins the non-active aldehyde derivative 5-hydroxyindoleacetic acid (5-HIAA) (Chase & Koelle, 2007; Curran & Chalasani, 2012). Several genes encoding the synthetic and recycling enzymes for the synthesis of co-factor tetrahydrobiopterin (BH4) for $TPH_2$ in the synthesis of 5-THP are also represented in figure 5.

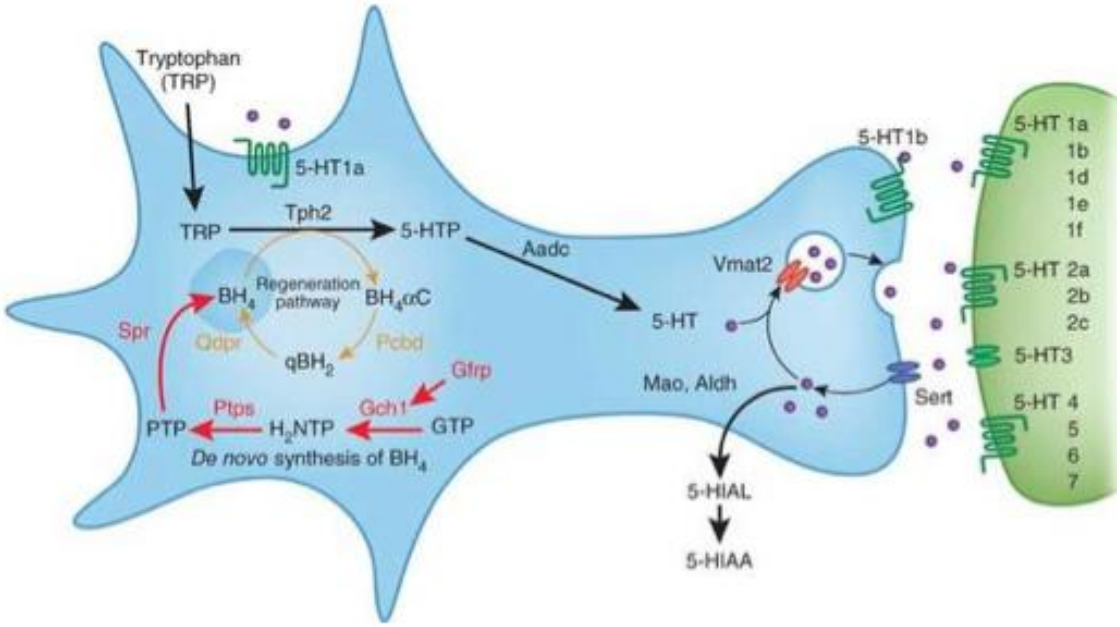


**Figure 5** – Synthesis and metabolism of serotonin. 5-HT neurons coexpress genes directing 5-HT synthesis (Tph2, Aadc), reuptake (Sert), vesicular transport (Vmat2), autoreceptor signaling (Htr1a, Htr1b) and metabolism (Maoa, Maob). Tetrahydrobiopterin (BH4), an essential cofactor for tryptophan hydroxylase 2 (Tph2) in the synthesis of 5-hydroxytryptophan (5-HTP), is synthesized (red pathway) de novo from guanosine triphosphate (GTP). It is also recycled through a regeneration pathway (orange). Genes Gch1, Gfrp, Ptps, Qdpr, Pcbd and Spr are directly involved in these BH4 red and orange pathways. Aldehyde dehydrogenase (Aldh) converts 5-hydroxyindolealdehyde (5-HIAL) into 5-hydroxyindoleacetic acid (5-HIAA). Synaptic 5-HT modulates 5-HT neuron firing through somatodendritic Htr1a autoreceptors (5-HT1a), 5-HT release from the presynaptic terminal through Htr1b autoreceptors (5-HT1b) and stimulates neurotransmission through postsynaptic 5-HT receptors (5-HT1–7). Aadc, aromatic l-amino acid decarboxylase; Sert, serotonin transporter; Vmat2, vesicular monoamine transporter 2; Maoa, monoamine oxidase; Gfrp, GTP cyclohydrolase I feedback regulator; Gch1, GTP cyclohydrolase 1; Ptps, 6-pyruvoyl-tetrahydropterin synthase; Spr, sepiapterin reductase; Pcbd, pterin-4-alpha-carbinolamine dehydratase; Qdpr, quinoid dihydropteridine reductase. BH4 synthetic intermediates: H2NTP, 7,8-dihydroneopterin triphosphate; PTP, 6-pyruvoyl-5,6,7,8-tetrahydropterin; BH4αC, tetrahydrobiopterin-4α-carbinolamine; qBH2, quinoid dihydrobiopterin (Taken from Deneris & Wyler, 2012).

Serotonin content in the central nervous system constitutes only 1–2% of the whole pool of this monoamine in the organism and it does not cross blood-brain barrier (Filip et al., 2005). Serotonergic CNS neurons are found primarily in nine clusters located mostly in the raphe nuclei of the midbrainm pons, and medulla (Figure 6a). The more rostral raphe nuclei contain the principal dorsal raphe groups (B6 and B7; depicted in yellow) and the median raphe groups (B5 and B8; depicted in green) that project to overlapping brain areas

structures, including amygdala, thalamus, hypothalamus, hippocampus, and frontal cortex. The more caudal nuclei (B1–B3) in the medulla project axons to the spinal cord and the periphery. On the other hand, in the periphery, a partially separate serotonergic enteric neural system (ENS) exists within the gut and interacts with other CNS and peripheral serotonergic mechanisms (PNS), that includes lung, heart, blood-vessel and pancreatic tissue, as well as platelets. Notably, these tissues normally take up and store serotonin in vesicles, and can release it in response to local stimuli (Figure 6b).

5-HT also play a role in the neuroendocrine serotonergic system (NSS) that includes the hypothalamo–pituitary–adrenocortical (HPA) system. There is an interaction between 5-HT and hormones such as prolactin, ACTH, corticosterone and oxytocin within this NSS system (Murphy & Lesch, 2008).

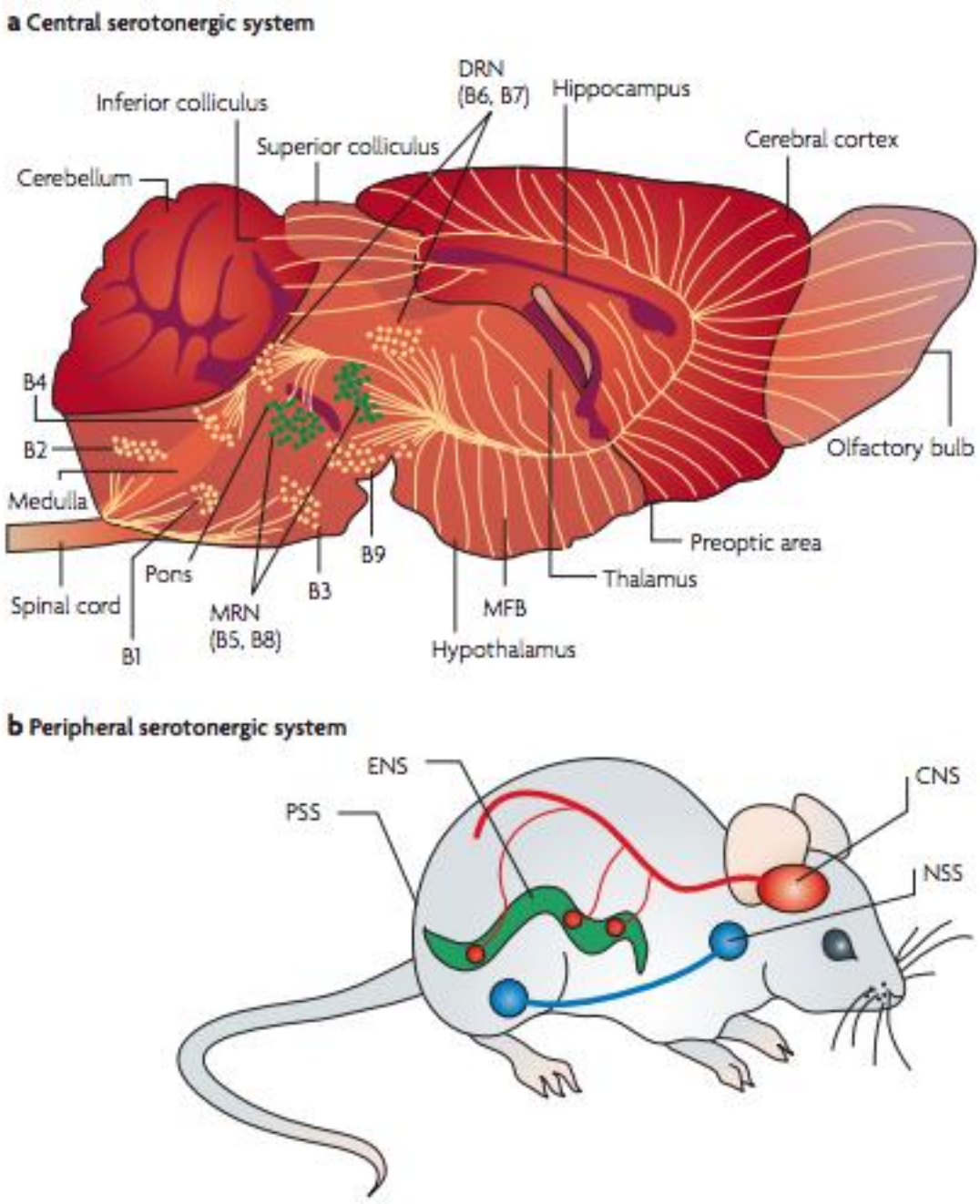


**Figure 6** – 5-HT pathways. **a** – **Central serotonergic system:** CNS serotonin neuron cell-body groups in the nine raphe nuclei, B1–B9. The more caudal nuclei (B1–B3) in the medulla project axons to the spinal cord and the periphery, whereas the more rostral raphe nuclei contain the principal dorsal raphe groups (B6 and B7; depicted in yellow) and the median raphe groups (B5 and B8; depicted in green), which project to different but overlapping brain areas. **b** – **Peripheral serotonergic system:** Serotonin also functions in the enteric nervous system (ENS), the hypothalamo–pituitary–adrenocortical (HPA) system, the adrenomedullary neuroendocrine serotonin system (NSS) and the peripheral serotonin system (PSS), which includes the lungs, the heart, the blood vessels, the pancreas and platelets. DRN, dorsal raphe nucleus; MFB, medial frontal bundle; MRN, median raphe nucleus. (Taken from Murphy & Lesch, 2008).

Therefore, central and peripheral 5-HT system fulfill a significant role in the regulation of many vital functions of the organism including sleep, circadian rhythm, emotional, feeding, cognitive and reproductive behaviors, thermoregulation, nociceptive transmission, motor, endocrine, cardiovascular and respiratory functions, and intestinal peristalsis. Moreover, serotonin plays a major role in the etiology of the related pathological states, including depression, anxiety, schizophrenia, obsessive –compulsive and panic disorders, autism, in addition to migraine, hypertension, pulmonary hypertension, eating disorders, vomiting and, irritable bowel syndrome (IBS) (Filip et al., 2005).

### 1.3.3. 5-HT Receptors

Based on structural (amino acid sequence), biochemical (postreceptor mechanisms of signal transduction) and pharmacological differences, 5-HT receptors were classified into 7 classes and 16 different subtypes. A majority of these receptors belong to the metabotropic receptor family, except for 5-$HT_3$ (5-$HT_{3A}$, 5-$HT_{3B}$ and 5-$HT_{3C}$) receptors, which are included in the ionotropic receptor family. 5-$HT_1$ (5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, 5-$HT_{1E}$ and 5-$HT_{1F}$) receptors inhibit adenylate cyclase; 5-$HT_2$ (5-$HT_{2A}$, 5-$HT_{2B}$ and 5-$HT_{2C}$) receptors stimulate phospholipase C; 5-$HT_4$, 5-$HT_6$ and 5-$HT_7$ receptors stimulate adenylate cyclase, while the mechanism of signal transduction via 5-$HT_5$ (5-$HT_{5A}$ and 5-$HT_{5B}$) has not been yet satisfactorily defined (Filip et al., 2005; Curran & Chalasani, 2012; Rojas et al., 2014).

Preclinical studies have shown an increase of 5-$HT_{1A}$ receptor-mediated hippocampal transmissions after long-term treatment with selective serotonin reuptake inhibitors (SSRIs) and other antidepressant drug classes, suggesting that this receptor is involved in mood disorders. However, most selective 5-$HT_{1A}$ agonists developed so far have failed to demonstrate clinical effectiveness (Celada et al., 2004). On the other hand, de Angelis (2002) reviewed data presenting evidence that there is an association between the 5-$HT_{2A}$ receptor gene and psychiatric disorders including schizophrenia, tardive dyskinesia, major depression, suicidality, anorexia nervosa and obsessive-compulsive disorder. Furthermore, chronic administration of 5-$HT_{2A}$ antagonists results in a paradoxical downregulation of 5-$HT_{2A}$ receptors. This was suggested to be of benefit in the treatment of depression (Glennon & Dukat, 2002). Also 5-$HT_{2A}$ receptors mediate the primary effects of hallucinogenic drugs (Vollenweider et al., 1998; Gonzalez-Maeso et al.,

2007). Interestingly, hallucinations and cognitive impairment are the typical clinical symptoms of METH psychosis.

### 1.3.4. Methamphetamine action in serotonergic systems

Serotonin has been shown to be of essential importance for the hedonic tone (O'Leary & Cryan, 2010), motivational (Lee & Clifton, 2010) and reinforcement processes (McBride, 2010), and for learning and memory (Cassel, 2010). Accordingly, it is not surprising that drugs of abuse, which induce profound changes in extra-cellular 5-HT activity and 5-HT receptor function, change the behaviour-organizing circuitry of the brain directly by modulating the 5-HT system, or, indirectly, by 5-HT effects on other transmitter systems (Adell et al., 2010). Chronic drug exposure alters 5-HT tissue levels, basal extracellular activity, and responsiveness of the 5-HT system to acute drug administration. These effects may serve as neurochemical mechanisms for the acute behavioural and subjective effects. They may also contribute to the transition to addiction.

Neurochemical studies showed that almost all psychoactive drugs with abuse potential acutely increase 5-HT activity throughout the brain (Muller & Homberg, 2014). In particular, it has been reported that METH acutely increases extracellular 5-HT levels in the nucleus accumbens (Nac), dorsal striatum, ventral hippocampus and prefrontal cortex (PFC) (Kuczenski et al., 1995; Segal et al., 1997; Rocher & Gardier, 2001; Ago et al., 2006; Ikeda et al., 2011 and Yubero-Lahoz et al., 2012) from rodents. Studies in rodents further showed that the rapid increase in the concentration of 5-HT METH-induced is mediated by different mechanisms: in addition to stimulating the release of 5-HT, it inhibits its reuptake (Brodkin et al., 1993) (Figure 7).

Neurotoxic METH doses following an acute aberrant 5-HT release also cause long-term deleterious effects to serotonergic neuronal systems (Krasnova & Cadet, 2009).

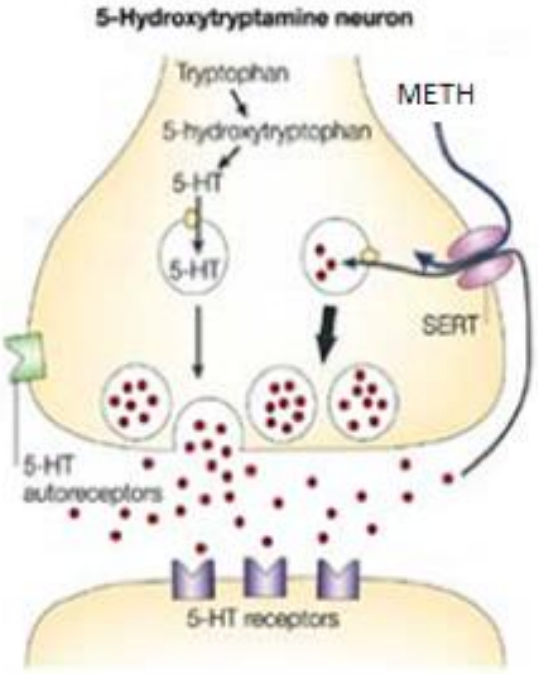


**Figure 7 –** The impact of METH on a serotonergic synapse (Adapted from Torres et al., 2003).

In fact, experimental neurotoxic rodent models including single-day multiple-dose and sub-chronic METH administration have consistently shown that dysfunctional serotonin nerve terminals are in place (Rusyniak, 2011; Marshall et al., 2007; Belcher et al., 2008 Ladenheim et al., 2000; Fumagalli et al., 1998). These serotonergic changes include a persistent fall in the levels of 5-HT as well as 5-HT transporter (SERT) within several brain regions including the striatum, hippocampus, medial prefrontal and somatosensory cortices, hypothalamus and amygdala (Kish et al., 2009; Sekine et al, 2006; Gluck et al., 2001; Friedman et al., 1998; Baldwin et al., 1993; Ohmori et al., 1993; Bakhit et al., 1981). Furthermore a significant decrease in SERT binding in the anterior cingulate, nucleus accumbens, amygdala, hippocampus, somatosensory cortex, hypothalamus, thalamus and septum was also reported (Armstrong & Noguchi, 2004; Guilarte et al., 2003).

Finally, these neurotoxic METH dosing regimens also disturb TPH activity in the cortex, hippocampus and nucleus accumbens from rodents (Bakhit et al., 1981; Hotchkiss & Gibb, 1980; Morgan & Gibb, 1980). These experimental models are consistent with terminal serotonergic toxicity measured in human addicts (see section *Depressive*

*behaviour during withdrawal from METH*). Moreover, Chiu et al. (2014) demonstrated that a single-day 'binge' METH dosing regimen caused an up-regulation of 5-$HT_{2A}$ receptor in the medial frontal cortex, a brain region involved in perception, cognition and mood. This is suggestive that this dosing regimen, while producing detrimental effects to serotonergic nerve terminals, also changes 5-HT receptor density.

It has also been shown that METH, can also cause other neurodegenerative changes that include dopaminergic terminal toxicity, loss of gray matter accompanied by hypertrophy of the white matter, gliosis (astrocytic and microglia proliferation and reactivity, in different brain areas), cortical and hippocampal BBB changes and oxidative stress (Krasnova & Cadet, 2010; Pereira et al., 2004, 2006, 2012; Bowyer & Ali, 2006; Bowyer et al., 2008; Martins et al. 2011, 2013; Silverstein et al., 2011; Ramirez et al., 2009; Park et al., 2012 and Moszczynska & Yamamoto, 2011).

# 2. Depression

Depressive disorders including major depressive disorder (MDD) rank within the most prevalent forms of psychiatric disorders and are among the leading causes of disability (Andrews et al., 2000). Today, depression is estimated to affect 350 million people (WHO, 2012; Wen et al., 2014) and has a 12-month prevalence of 6.6% and a lifetime prevalence of 16.2%, is twice as common in women as in men, and causes considerable impairment (Kupfer et al., 2012; Belmaker & Agam, 2008). The World Health Organization (WHO) estimates that depression will be the second most prevalent cause of illness-induced disability by the year 2020 (Murray & Lopez, 1997).

Depression presents with depressed mood, loss of interest or pleasure (anhedonia), irritability, decreased energy, feelings of guilt or low self-worth, disturbed sleep or appetite, and poor concentration (Warner-Schmidt & Duman, 2006). Moreover, depression often comes with symptoms of anxiety. These problems can become chronic or recurrent and lead to substantial impairments in an individual's ability to take care of his or her everyday responsibilities. MDD is the leading cause of suicide worldwide. Almost 1 million lives are lost yearly due to suicide, which translates to 3000 suicide deaths every day. For every person who completes a suicide, 20 or more may attempt to end his or her life (WHO, 2012; Wen et al., 2014). Most likely, depression is caused by a combination of genetic, biological, environmental, and psychological factors. Thus, vulnerable and/or exposed individuals to certain endogenous and exogenous stressors, may develop, cognitive, behavioral and somatic emotional dysfunction that translates into a depressive syndrome (Tsuang et al. 2004).

Although the identification of specific neural substrates for depressive disorders is still under investigation, hippocampus has received a great deal of attention (Warner-Schmidt & Duman, 2006). Particularly, preclinical and clinical studies demonstrate that reductions of the total volume of neurons and neuronal atrophy or loss occur in the adult hippocampus in a context of stress and depression. Hippocampal circuitry functions include the control of learning and memory and regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which are altered in depression. In addition, the hippocampus interacts with the amygdala and frontal cortex, regions that are more directly involved in emotion cand cognition and thereby contribute to major symptoms of depression (Duman & Monteggia, 2006).

## 2.1. Depression: Focusing on 5-HT

The 5-HT hypothesis of depression stretch back over 40 years to the discovery that the first generation of antidepressant drugs blocked 5-HT reuptake or metabolism as part of their pharmacological effect (Sharp & Cowen, 2002). Although the nature of the 5-HT defect remains elusive, the best evidence that 5-HT contributes to the pathophysiology of depression comes from studies of tryptophan depletion, which show that lowering brain 5-HT levels can induce acute symptomatic relapse in recovered depressed patients (Cowen, 2008; Ruhé et al., 2007; Smith et al., 1997).

This link between 5-HT and depression provided a rich source of discoveries including the development of newest and popular selective 5-HT reuptake (SSRIs), which are currently the first choice antidepressant drug treatment. SSRIs block 5-HT reuptake, leading to an increase in extracellular 5-HT levels (Blier, 2001; Quesseveur et al., 2013). This monomine increase activates feedback mechanisms mediated by 5-$HT_{1A}$ (cell body) and 5-$HT_{1B}$ (terminal) autoreceptors, which, respectively, reduce the firing in dorsal raphe 5-HT neurons and decrease the amount of 5-HT released per action potential, resulting in attenuated 5-HT neurotransmission in regions such as frontal cortex and hippocampus. Long-term treatment desensitizes the inhibitory 5-$HT_1$ autoreceptors, and 5-HT neurotransmission is enhanced. The time course of these events is similar to the delay of clinical action.

Overall SSRIs seem to allow what might be an important convergence of neurobiological and psychological explanations of the role of 5-HT in antidepressant action and depression (Sharp & Cowen, 2002). The oldest class of antidepressant medications is monoamine oxidase inhibitors (MAOIs). Tricyclics are other types of older antidepressants. Tricyclics antidepressants (TCA) inhibit non-selectively 5-HT and noradrenaline uptake (Katzung et al., 2001). Interestingly, previously referred hippocampal alterations can be reversed by chronic antidepressant treatment (Lee & Kim, 2010). However the nature of post-synaptic signaling and the contribution of glia to the antidepressant pharmacological effects are largely unknown. Both TCAs and MAOIs are potentially lethal in overdose, require titration to achieve a therapeutic dose, have serious drug interactions and have many troublesome adverse effects. Consequently, these drugs are now reserved for patients who do not respond to other agents.

Moreover, due to its long therapeutic time delay, low rates of remission, and exposure to relevant side effects, including gastrointestinal, hepatic and sexual, the

research for more effective agents in the treatment of these disorders has been greatly stimulated (Manji et al., 2008; Berton & Nestler, 2006). For example, physical exercise, being able to relieve symptoms of depression (APA, DSM-V-TR, 2013), seems to be a non-pharmacological alternative strategy in the treatment of depression.

Finally, although not addressed in our work, other hypothesis pointing towards a role for other neurotransmitters including NA, inflammation, neuroplasticty and epigenetic regulation have also been implied in depressive disorders (Han & Yun, 2014; Massart et al., 2012; Krishnan & Nestler, 2008).

## 2.2. Depressive behaviour during withdrawal from METH

Mood disorders, including depression and bipolar disorders, are the most common psychiatric comorbidities among patients with substance use disorders (Quello et al., 2005; NIH, 2011). For example, diminished interest or pleasure in rewarding stimuli or anhedonia is one of the core symptoms of both depression and psychostimulant withdrawal. Importantly, METH withdrawal and major depression share many behavioral commonalities in humans (Cryan et al., 2003). In detail, repeated METH use produces a withdrawal syndrome marked more by psychiatric complaints than by physical manifestations. These withdrawal syndrome typically manifests as anxiety, depression with severe dysphoria, irritability and melancholia, social isolation, fatigue with hypersomnia, psychomotor dysfunction, mood disturbances, impaired social functioning, intense craving for the drug and even paranoia or aggression, as well as attention deficits and memory in making decisions (London et al., 2004; Meredith et al., 2005; Darke et al., 2008; Homer et al., 2008, Sutcliffe et al., 2008; Krasnova & Cadet, 2009; Daniulaityte et al., 2010; Guerreiro & Carmo et al., 2011).

Although the severity of the abstinence syndrome appears to be related to the frequency of use, it tends to resolve spontaneously, while the depressive symptoms persist for months or years after discontinuation of drug use (Barr et al., 2002; Cami & Farre, 2003). Moreover, the prevalence of depression among METH users is higher than the general population, with the majority of METH users reporting a lifetime history of depression and over a third reporting a lifetime diagnosis of depression. However, the temporal relationship between depression and METH use is unclear. In fact, it is unknown whether experiencing depressive symptoms promotes METH use, whether depression

results from or is enhanced by METH use, or whether it is bidirectional (Sutcliffe et al., 2009).

Addressing this issue is clinically relevant as it may inform about treatment options for both METH dependence and mental health (Pecke et al., 2005). Therefore, the examination of the behavioral effects of METH withdrawal in rodents may provide insights into the neurobiological mechanisms underlying both disorders. For example, Cryan et al. (2003) showed that drug-withdrawn rodents displayed increased immobility in forced swim and tail suspension tasks (24 hours after deprivation of METH; 5 mg/kg/day for 7 days), suggesting that there is a depressive-like behavior. Recently, Jang et al. (2013) found that chronic METH self-administration produced a depressive-like withdrawal state in rats during early withdrawal.

We also recently provided novel data that a single high neurotoxic dose of METH (30 mg/kg i.p.) evoked depressive-like behavior as gauged by increased immobility in the tail-suspension task at 3 days and 7 weeks post-METH treatment (Silva et al., 2014). This negative affect-like behavior was underlined by prolonged frontostriatal dopaminergic deficits including DA and TH. Importantly we also demonstrated that this METH regimen evoked 5-HT depletion in frontal cortex at 3 and 49 days post-METH injection. However we failed to probe METH impact in hippocampus. This is suggestive that this 5-HT homeostasis disruption might also contribute to the depressive-like behavior.

Interestingly, abstinent METH abusers showed reduced brain 5-HT transporter density and cerebral metabolic abnormalities associated with depressive symptoms (London et al., 2004; Sekine et al., 2006; Panenka et al., 2012). It is worthwhile stress that psychostimulant withdrawal seems to provide the basis for the development of an animal model of depressive symptoms, such as despair, anhedonia, lethargy and anxiety (Paulson et al., 1991; Cryan et al., 2003), thus allowing the screening of new pharmacological or non-pharmacological approaches (Barr et al., 2002). This is particularly relevant since there is no reliable treatment that can reverse the effects of psychostimulant withdrawal rapidly and completely at present.

# 3. Physical Exercise

## 3.1. Physical Exercise: a non-pharmacological approach in depressive disorders

Data from epidemiological studies suggest a strong association between physical inactivity and high levels of depressive symptoms (Farmer et al., 1988; Camacho et al., 1991; Conn et al., 2010). In fact, low levels of physical activity lead to an increase in symptoms of depression in young (Motl et al., 2004; Strong et al., 2005; Neissaar et al., 2011) and older adults (Blumenthal et al., 1999; Lampinen et al., 2000; Brosse et al., 2002; Conn et al., 2010).

On the other hand, human studies suggest that exercise may be useful in the prevention and treatment of psychiatric disorders such as depression (Conn et al., 2010) and anxiety disorders (Dunn et al., 2010), Furthermore, Lawlor & Hopker (2001) reviewed data suggesting that exercise has antidepressant effects that are of the same magnitude as cognitive therapy in the management of mild-to-moderate mental health diseases. It also was demonstrated that the antidepressant effects of exercise were prolonged beyond the period of treatment with benefits until 6 (Babyak et al., 2000) and 21 months (Singh et al., 2001) after stopping the exercise. Trivedi et al. (2006) showed that exercise reduced depressive symptoms or reduced side effects when combined with a pharmacological approach. In addition, Frazer et al., 2005 indicate chronic exercise as one of the best non pharmacological treatments for depression, besides the fact that it is low cost, have positive effects on cardiovascular disease risk, and be a potential prevention agent for future depressive episodes. Others further proposed that exercise was as effective as antidepressant medications (Blumenthal et al., 1999; Strawbridge et al., 2002). However, habitual physical activity has not been shown to prevent the onset of depression (Paluska & Schwenk, 2000). One possible neurobiological mechanism underlying the positive effects of exercise is the increased synthesis of 5-HT (Matta et al., 2013). This is the studied hypothesis in the present work.

Despite the relationship between the efficacy of physical exercise in reducing the symptoms of depression already has been widely documented, there is still a lack of data on the type of physical exercise that induces better antidepressant response (Mead et al., 2008). On one hand, aerobic exercise is associated with higher benefits (Ernst et al., 2006). For example, a program of 12 weeks of aerobic exercise functioned as an effective

treatment for depression of mild to moderate severity (Dunn et al., 2002, 2005). Furthermore, increased aerobic exercise or strength training has been shown to significantly reduce depressive symptoms (Paluska & Schwenk, 2000; Craft & Perna, 2004). On the other hand, Greenwood et al. (2013) were the first to suggest that individuals who have made forced physical exercise can still benefit from its protective effects against psychiatric disorders related to stress.

Although effective pharmacological interventions are available, depression remains inadequately treated. Thus, physical exercise seems to be a non-pharmacological tool in the treatment of psychiatric diseases such as depression and, therefore, in the promotion of a better mental health.

A cautionary note should be added. In fact, it was suggested that excessive physical activity may lead to overtraining and generate psychological symptoms that mimic depression (Paluska & Schwenk, 2000).

## 3.2. Physical Exercise: managing METH addiction and neurotoxicity

To date, there are no medications approved for the treatment of METH dependence (Mooney et al., 2014 and Karila et al., 2010). Behavioral approaches, such as cognitive behavioral therapy and contingency management, have proven modestly effective and remain the standard treatment. However, there are substantial proportions of individuals dropping out early in the treatment (Rawson et al., 2004; Peirce et al., 2006; Roll et al., 2006; Rawson et al., 2006).

Notably, there is an increasing amount of literature suggesting that physical exercise is as a potential treatment for METH-dependent individuals (Mooney et al., 2014). For example, it was demonstrated that physical exercise improved cognitive deficits found in chronic METH users (Simon et al., 2000). Recently, a study in humans reported for the first time that 8-weeks of endurance and resistance exercise training triggered positive adaptations on individuals recovering from METH-dependence, such as substantial improvements in aerobic exercise performance, muscle strength and endurance, and body composition with exercise training. These changes are consistent with those seen in the general population, suggesting that structured and supervised exercise training should be considered as part of the overall treatment plan in recovering METH patients (Dolezal et al., 2013). Another study examined whether a combination of aerobic exercise and

resistance training could facilitate relapse prevention and maintenance of abstinence from METH after discharge from residential treatment (Mooney et al., 2014). These authors suggested that exercise may provide a reinforcing behavior that offers an alternative to drug use as a means of enhancing positive mood states.

Furthermore, exercise may have a salutary effect on reducing cardiovascular risk factors, such as hypertension, that are associated with METH use (Mooney et al., 2009; Turnipseed et al., 2003). Exercise also improves sleep (Youngstedt et al., 2005) and performance on cognitive tasks, which may be impaired in METH users and in long-term METH users in early phases of abstinence (Mooney et al., 2014).

On the other hand, O'Dell et al. (2012) demonstrated that running wheel exercise ameliorates methamphetamine-induced damage to forebrain DA and 5-HT terminals in rats. Moreover, it was shown that exercise protected against cerebrovascular toxicity of METH mice (Toborek et al., 2009). Remakably, we have recently shown that treadmill exercise reversed the long-lasting behavioural phenotype triggered by single-METH neurotoxic dose as demonstrated by decreased immobility time in the tail suspension test when compared to sedentary METH-intoxicated mice (Pereira et al., 2014, unpublished data).

## 4. Aims of this thesis

This study aims to further characterize the long-lasting depressive phenotype caused by a single neurotoxic METH-injection and to probe for a correlation between frontal-cortical and hippocampal 5-HT homeostasis and e the antidepressant effect of physical exercise.

# CHAPTER 2

# MATERIAL AND METHODS

# 1. Animals

In this investigation we used 44 C57BL/6 male mice (10 weeks old, 21-26 g, Charles River Laboratories, Barcelona, Spain) (Figure 8), divided into 11 cages (4/cage) and maintained in the animal house of the Faculty of Medicine, University of Coimbra (FMUC) under controlled environmental conditions (temperature, 22 ± 1 ° C, humidity 50 ± 10 %, light cycle, 12/12 hours), with food and water provided *ad libitum*. Their weight was monitored weekly to assess the evolution of the different experimental groups.

All experimental procedures followed the rules imposed by the Standards of Animal protection techniques used for experimental and other scientific purposes (Ordinance No 129/92 of 6 July), as well as the standards of the European Convention on Animal Welfare (Ordinance No 1005/92) and in accordance with the guidelines of the European Community (2010/63/EU). All efforts to minimize animal suffering and to use the smallest possible number of animals were made.

**Figure 8 –** Example of a C57BL/6 mouse. Taken from http://jaxmice.jax.org/strain/002020.html.

# 2. Drugs and Chemicals

We were issued permission to import methamphetamine.HCL (METH) for Sigma-Aldrich (St. Louis, MO, USA9 by INFARMED, Portugal (National Authority of Medicines and Healths Products). Standards for serotonin (5-HT) were purchased from Sigma-Aldrich. The other used chemicals (ultrapure and pro analysis quality) were purchased from Sigma-Aldrich and Merck AG (Darmstadt, Germany).

# 3. Experimental Design

32 animals were randomly divided into 4 groups, with 8 animals per group: (1) sedentary saline group (Cont/SED), (2) exercise saline group (Cont/EX), (3) METH sedentary group (METH/SED) and (4) exercise METH group (METH/EX) (table 2). The animals were previously identified with ink stripes on the tail and housed in their respective cages, having been subjected to an adaptation period of 2 weeks in controlled vivarium conditions (mentioned above). The weight of the mice was recorded weekly, using an analytical balance (Kern CB 6 K1, Germany). However, the weighing was done daily on subsequent administration of the neurotoxin week.

**Table 2** – Experimental groups and number of mice used (n).

| **Mice (n=32)** | | | |
|---|---|---|---|
| **Exercise (n=16)** | | **Sedentary (n=16)** | |
| METH/EX **(n=8)** | Cont/EX **(n=8)** | METH/SED **(n=8)** | Cont/SED **(n=8)** |

## 3.1. Treadmill adaptation

Herein, two treadmills were used [the LE8700 model, serial number 2187/07 and the LE8706 model, serial number 8589/04, both with 50 W, 110/120 V and 50/60 Hz; Panlab, SL, Barcelona, Spain,] for the implementation of aerobic exercise. Separating each lane with transparent acrylic, thus diminishing the width of each corridor and resized these models, which are suitable for mice. Therefore, it was possible to exercise 4 mice per treadmill at the same time (Figure 12).

Mice submitted to exercise (SAL/EX and METH/EX groups) were putin a daily workout plan aiming to foster treadmill adaptation for 2 weeks (5 days/week). The adaptation protocol consisted in four phases (Figure 9), with a progressive increase in the total time (20 min at the beginning to 40 min on the last week) and intensity (daily maximum speed of 20 to 30 cm/s) of each training session until achieving the values used in the exercise protocol.

The treadmill was turned on to a minimum speed of 5cm/seg only after and the animals were placed in the lanes. Thereafter, the speed was gradually increased (about 1cm/seg) until the desired speed, thus comprising the initial stage of the session - heating

phase. After the time and intensity required for the training session were applied, the speed periodically decreased at the same rate until the treadmill is turned off -– cooling phase. Both phases are relevant because they aim to prevent injury.

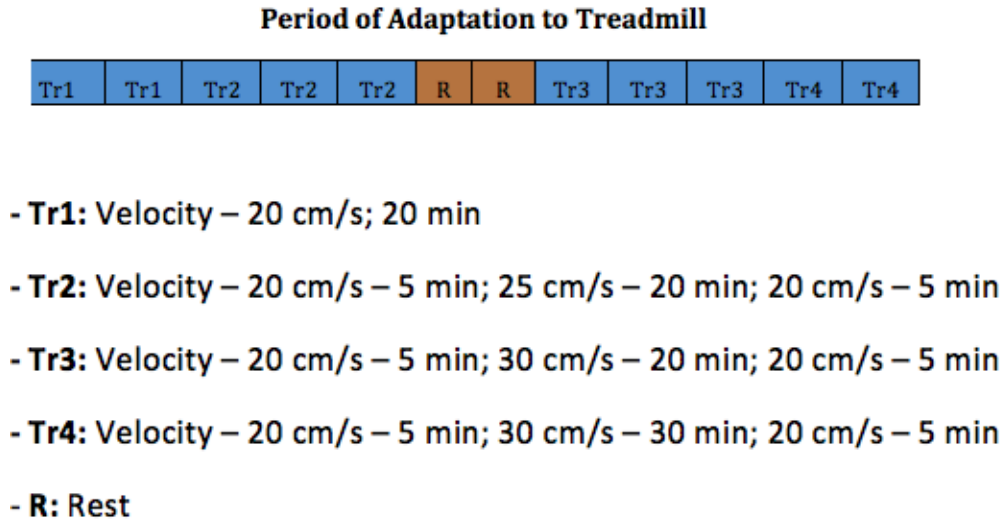


**Figure 9 –** Mice adaptation protocol to treadmill. Tr, training and R, rest.

## 3.2. Methamphetamine administration

Subsequently to the adaptation protocol to the treadmill and prior to the beggining of the exercise protocol, all mice were weighed again to calculate METH dose for each mouse.

Control groups (SAL/SED and SAL/EX groups) were administered a saline solution (NaCl 0.9%, 250µl.), while METH/SED and METH/EX groups were injected with 30 mg/kg methamphetamine.HCL (METH; 3mg/ml). Animals were dosed with a single intraperitoneal injection (i.p.) (Figure 10). We have recently demonstrated that this dose induces astrogliosis, as well as dopaminergic and serotonergic terminal toxicity to striatal-cortical regions in mice as soon as 3 days post-METH (Pereira et al. 2012; Silva et al. 2014). However, we failed to characterize serotonergic homeostasis in hippocampus from these neurotoxic models at this time-point. Therefore we used another set of 12 mice to probe METH-impact on hippocampal total 5-HT levels at 3 days post-injection. Half of the animals were injected with saline.

**Figure 10** – Intraperitoneal administration of a single dose of METH (30 mg/kg).

After injection, the animals were carefully observed, at 0.5, 1 and 3 time-points. Animals receiving this neurotoxin were extremely agitated, which is consistent with what is reported in the literature (Meredith et al., 2005).

Two of the mice belonging to the METH/EX group were found dead the following day. This may be due to hyperthermia caused by METH.

Infarmed Portugal (National Authority of Medicines and Health Products IP) issued necessary authorizations for FMUC to acquire METH from Life Science and High Technology Company Sigma-Aldrich (St. Louis, MO, USA).

## 3.3. Exercise protocol

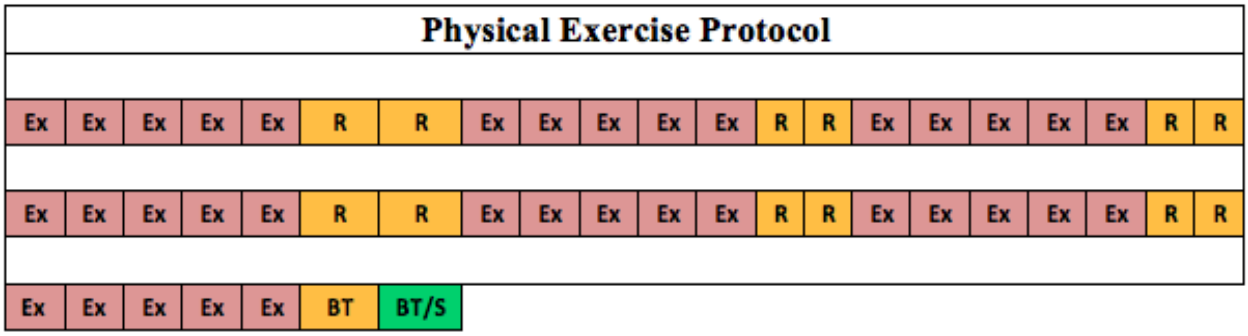


**Figure 11** – Physical exercise protocol for the respective exercise groups, SAL/EX and METH/EX. Ex, exercise; R, rest and S, sacrifice.

Twenty-four hours follwoing METH/NaCl administration, the exercise groups (SAL/EX and METH/EX) underwent a protocol of daily running (Figure 11), 5 days per week for 7 weeks (35 days of exercise) according to the following planning: 1) 5 min heating at 20 cm/s; 2) 30 min of running at 30 cm/s; 3) 5 min cooling at 20 cm/s. Exercise was always performed on the same morning period. The inclination of the treadmill was always null (0%). Mice were prompted to run by gentle manual strokes instead of using electric shocks, thus minimizing stress imposed to animals, which is considered to be a possible factor stress that is not usually associated with exercise.

**Figure 12 –** Mice training on a treadmill separated by acrylic divisions, providing four individual lanes.

The animals in the sedentary groups ran only once a week for 10 minutes at minimum speed of 5 cm/s to experience the same stressful conditions felt by the animals of the exercise groups. Woods et al. (2003) proposed that the engine noise of the treadmill, vibration, treadmill texture, water and food deprivation and frequent handling of animals are some of the stressful factors.

## 4. Behavioural Tests

Behavioural tests, Open-field (OF), Splash and Forced-swim test (FST) were performed, 48 and days after administration of METH (30 mg/kg, i.p.) or saline to assess mice emotional states. The first test was performed in the first day, whereas the Splash and the FST were conducted on the second day.

Tests were all performed between 9 and 17h, in a sound-attenuated room lit with low intensity Light (12 lx). Mice were transferred to this room 1h before the start of the testing to get used to the environment. The behaviour was monitored by a video camera positioned above the equipment and the images were subsequently analyzed with the system of video monitoring ANY Maze (Stoelting Co., Wood Dale, IL, USA) by an experienced experimenter who was unaware of the experimental group being tested.

## 4.1. Open-field test

The open-field (OF) test procedure consists of subjecting an animal to an unknown environment from which escape is prevented by surrounding walls (Walsh and Cummins, 1976). We applied herein a 5-min test length to assess the effect of treadmill exercise on novel environment exploration and general locomotor activity in METH-treated METH mice (Bayley & Crawley, 2009; Machado et al., 2012.

Mice were individually placed in the center of a wooden box (40×40×50 $cm^3$), and the following behavioral items were recorded: horizontal locomotion (total distance travelled), central distance, central-total distance ratio, number of rearing (sometimes termed vertical activity) and average speed. Tipically, mice prefer spontaneously the periphery of the apparatus to activity in the central parts of the open field (Figure 13). Indeed, rodents walk close to the walls, a behavior called thigmotaxis. Therefore, increase of time spent in the central part, as well as of the ratio central/total locomotion are indications of anxiolytic-like behavior (Prut & Belzung, 2003).

The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

**Figure 13 –** Open Field test: mice in open-field test apparatus.

## 4.2. Splash test

This pharmacological test was adapted from Yalcin et al. (2005) and was used herein to evaluate the impact of treadmill exercise on grooming behaviour in mice injected with METH as an index of self-care and motivational behaviour phenotype of the experimental groups. For this purpose, 10% sucrose solution was squirted on the dorsal coat of mice in their home cage. Following this viscous solution dirtying the mice fur, animals initiate grooming behaviour. The latency for the first grooming and the total grooming time were recorded in an acrylic chamber (40 x 40x 40 cm) during 5 minutes (Figure 14).

Grooming bouts were recorded including nose/face grooming (strokes along the snout), head washing (semicircular movements over the top of the head and behind the ears) and body grooming (body fur licking) (Kaluelf & Tuohimaa, 2004). Anhedonic symptoms were characterized by increased latency (midle time between spray and initiation of grooming) and decreased time spent grooming (d'Audiffret et al., 2010).

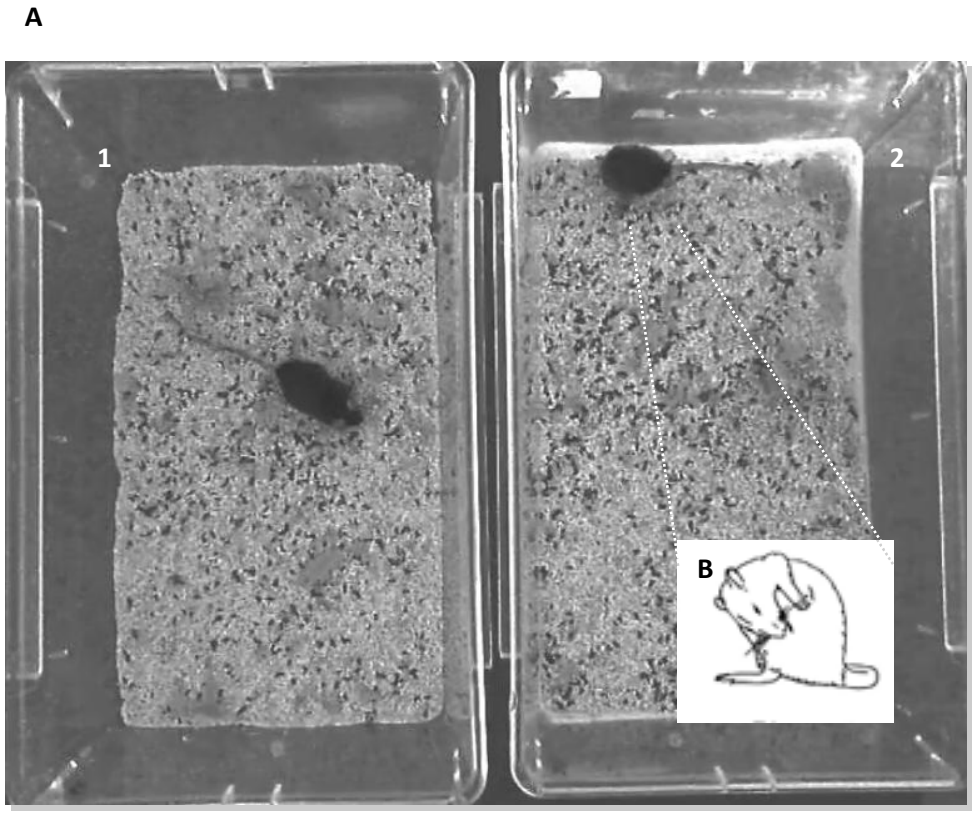


**Figure 14** – Splash test: **A** - mice in the cage 2 is grooming his dorsal coat in his home cage; **B** - schematic illustration of dorsal mice grooming. Adapted from http://mybrainnotes.com/bdd-trichotillomania-skin-picking.html.

## 4.3. Forced-swim test

The Forced-swim test (FST) has been used as a model predictive of antidepressant effect (Cryan et al., 2002). The test procedure was carried out according Porsolt et al. (1977) with some modifications. Mice were individually forced to swim in an open cylindrical container (21 cm height × 12 cm internal diameter) containing fresh water till a height of 15 cm at 23±1 °C; the total duration of immobility was recorded during a 5 minute period (Figure 15). The water was changed and the cylinder rinsed with clean water after each mice. A trained observer that was blind to the treatment measured the immobility time.

During the 5 min swimming test session, the following behavioral responses were recorded by a trained observer: the immobility time (i.e. the time spent floating in the water without struggling, making only those movements necessary to keep its head above water), swimming (time spent actively swimming around in circles), and climbing behavior, which is defined as upward directed movements of the forepaw along the cylinder walls. This test involved the scoring of active (swimming and climbing) and passive (immobility) behavior when mice were forced to swim in a cylinder from which there is no escape. A decrease in the duration of immobility time and an increase in swimming time is indicative of an antidepressant-like effect (Porsolt et al., 1977), while time of climbing was used as a predictor of altered motor activity scored directly in the forced swimming test (Vieira et al., 2008). Typically, after the initial 2-3 minutes of vigorous activity the animals showed a period of immobility by floating with minimum movements. An animal is considered to be immobile whenever it remained floating passively in the water in a slightly hunched but up right position, its nose above the water surface (Khulbe et al., 2013).

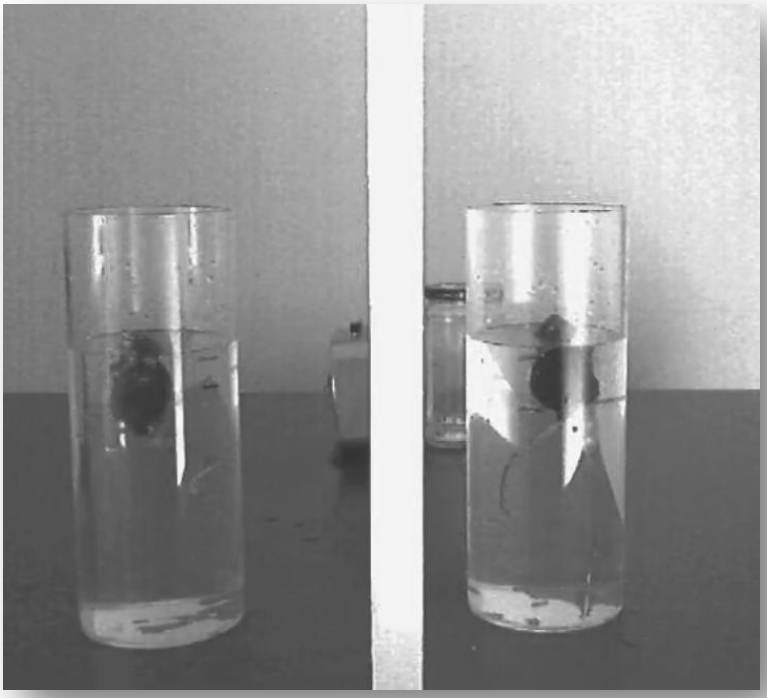

**Figure 15 –** Forced-swim test. Mice placed into an open cylindrical container.

## *5.* Frontal Cortex and Hippocampus Isolation

Following behavioural tests, animals were sacrificed by cervical dislocation and decapitated - that is 48 h after the end of the protocol of physical exercise (on the second day of rest) or 49 days after administration of METH/saline.

The brains were rapidly removed, and hippocampus and the frontal cortex were dissected on icebased on the coordinates for the mouse brain described by Paxinos & Franklin (2004). The biological samples were immediately frozen in dry ice and stored at -80 ° C until 5-HT analysis by high pressure liquid chromatography with coulometric electrochemical detection (HPLC).

## 6. Determination of 5-HT by HPLC

A reversed-phase high-performance liquid chromatography (HPLC) method with coulometric electrochemical detection was applied to quantify 5-HT brain levels (Figure 17) (Pereira et al., 2011). The used equipment is illustrated in figure 16 and included a Gilson pump (model 307), an autosampler Gilson (model 234; 50mL loop), a detector Gilson (coulometric) and v5.11 software.

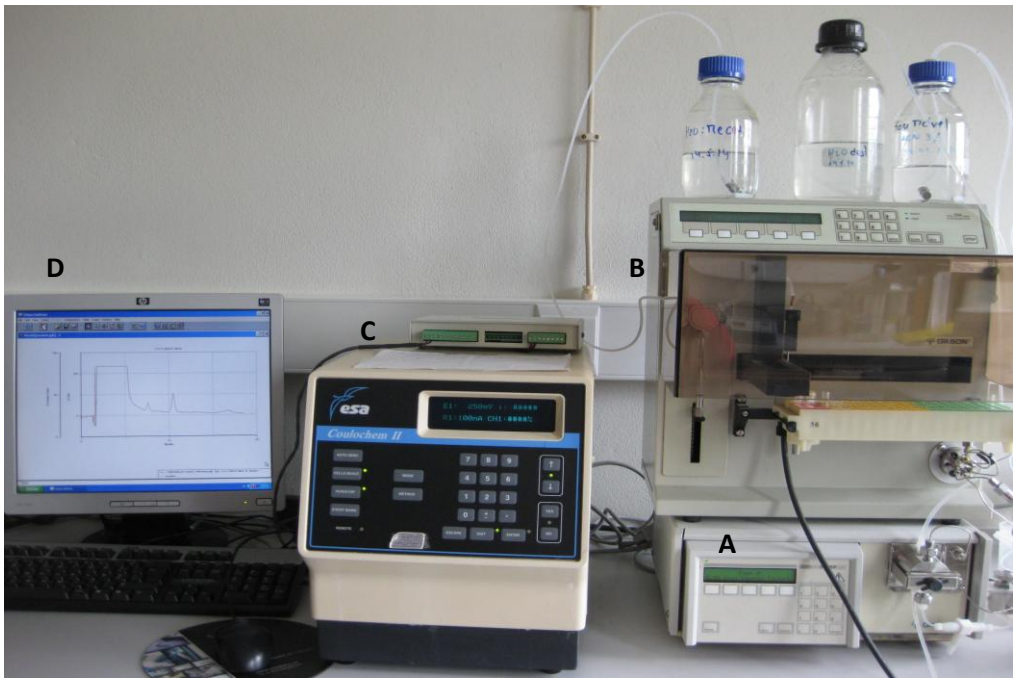


**Figure 16 –** HPLC system used to quantify 5-HT in this study. A - Gilson pump, B - autosampler Gilson, C - detector Gilson and D - v5.11 software. (Picture taken at Laboratory of Pharmacology and Experimental Therapeutics, Faculty of Medicine, University of Coimbra).

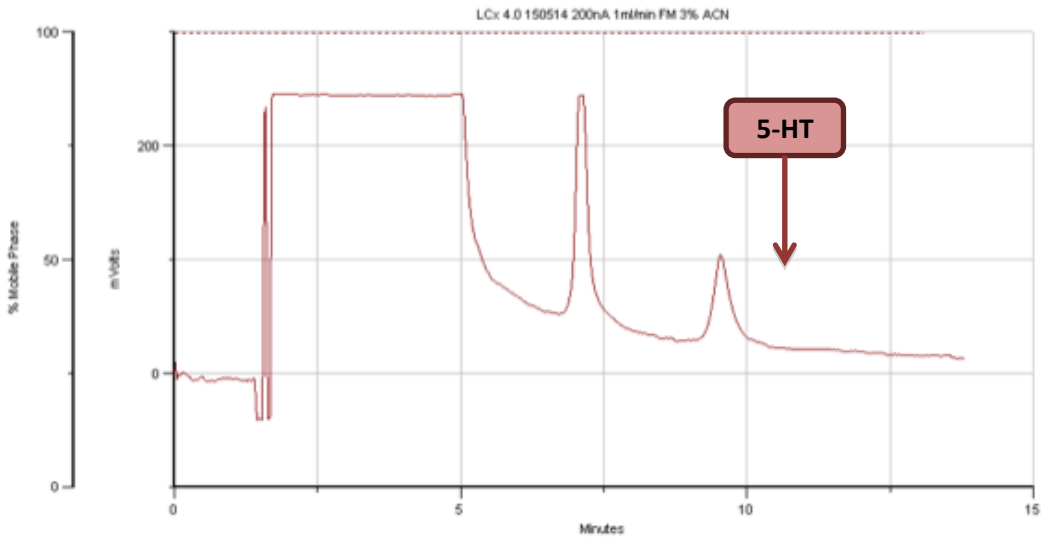


**Figure 17** – Chromatogram illustrating a 5-HT peak relative to a frontal cortex sample from a METH/EX mice group. The obtained 5-HT retention time was between 8 - 9 min.

Left Hippocampi and frontal cortices were thawed and homogenized in 250 □l perchloric acid 0.2 M. The homogenates were centrifuged (13,000 rpm for 7 min, 4 ° C) and filtered with 0.22 mM Nylon microfilter (Spin-X ® Centrifuge Tube Filter, Costar at 10,000 rpm for 4 minutes, 4° C). The pellets were resuspended in NaOH 1 M and used for quantification of the protein by the bicinchoninic acid method (BCA) and reading by ELISA. The filtered supernatants and the pellets were stored at -80 ºC.

Serotonin was separated on a reversed-phase, Grace Platinum EPS C18 column ® (4.6 x 150 mm, particle size: 5 mM) with a mobile, degassed and filtered phase consisting of monopotassium phosphate (25 mM), 1-heptanesulphonic acid (0.4 mM), EDTA (50 µM), 3% acetonitrile (pH 2.5). The flow rate was 1 mL/minute. Samples were quantified by an analytical cell (model 5011, ESA Analytical, Dorton Aylesbury, Buckinghamshire, UK) attached to a Coulochem-II electrochemical detector (ESA, Analytical). The analytical cell was set at E1=+250 mV (sensitivity - 200 nA).

The concentration of 5-HT in each sample was calculated taking as reference the standard curves of 5-HT. The results were expressed in ng/mg protein.

## 7. Statistical analysis

The results are expressed as mean ± standard error (SEM). Body weight data were analysed by one-way ANOVA followed by Newman–Keuls multiple comparison.

Behavioural and neurochemical data were analysed by two-way ANOVA (drug x exercise) followed by Bonferroni multiple comparison. Significant differences were defined at $P<0.05$. The statistical analyses were performed using GraphPad Prism 6.0 software.

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# CHAPTER 3

# RESULTS

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# 1. Effects of methamphetamine and/or chronic physical exercise on body weight

The mice body weight significantly increased in all groups ($P<0.05$) over the total duration of the experimental protocol (61 days, Figure 18 A,B). Interestingly, mice body weight from each group is not significantly different from each other at the last time evaluated ($P> 0.05$) (Figure 18 B).

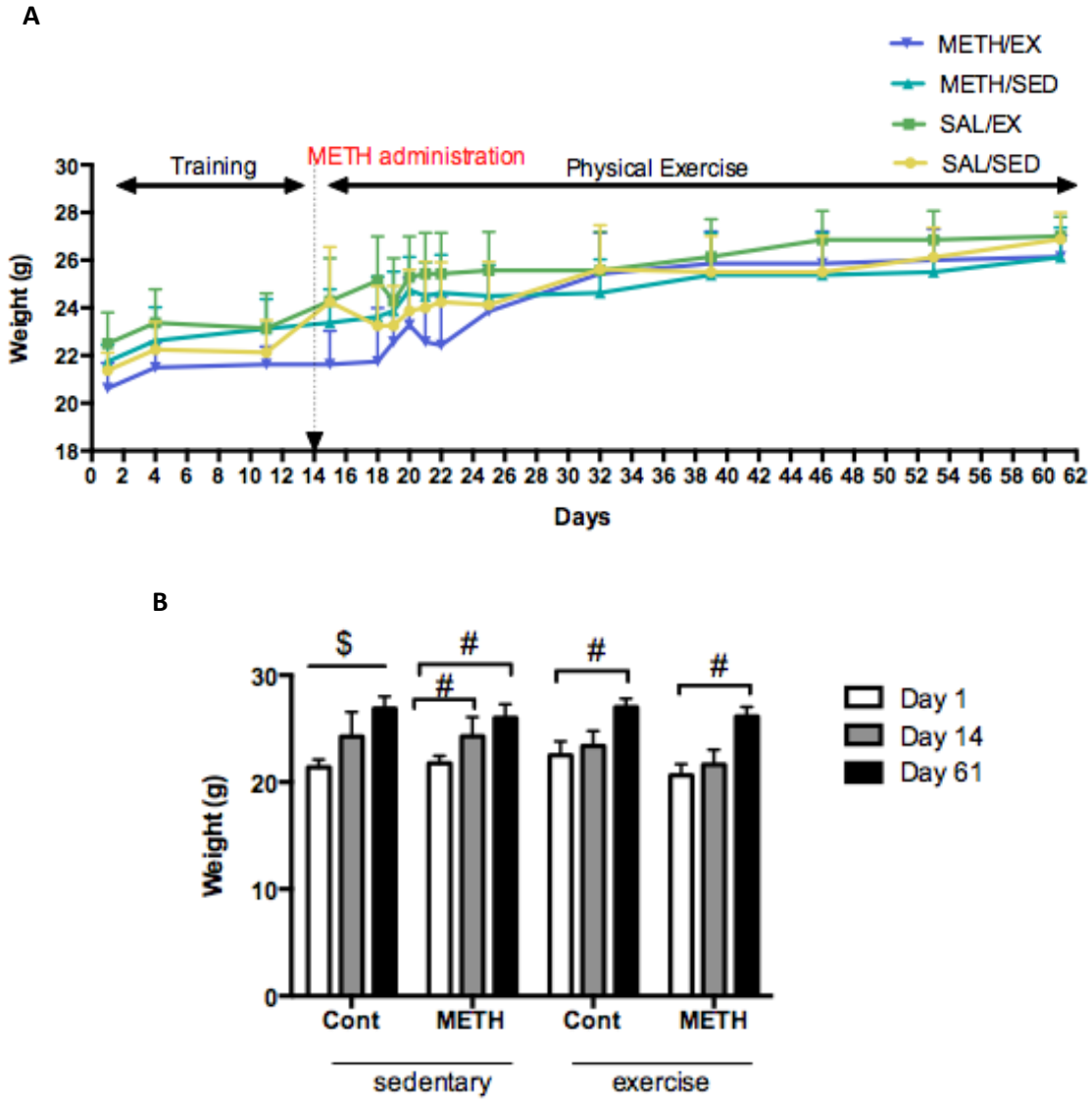
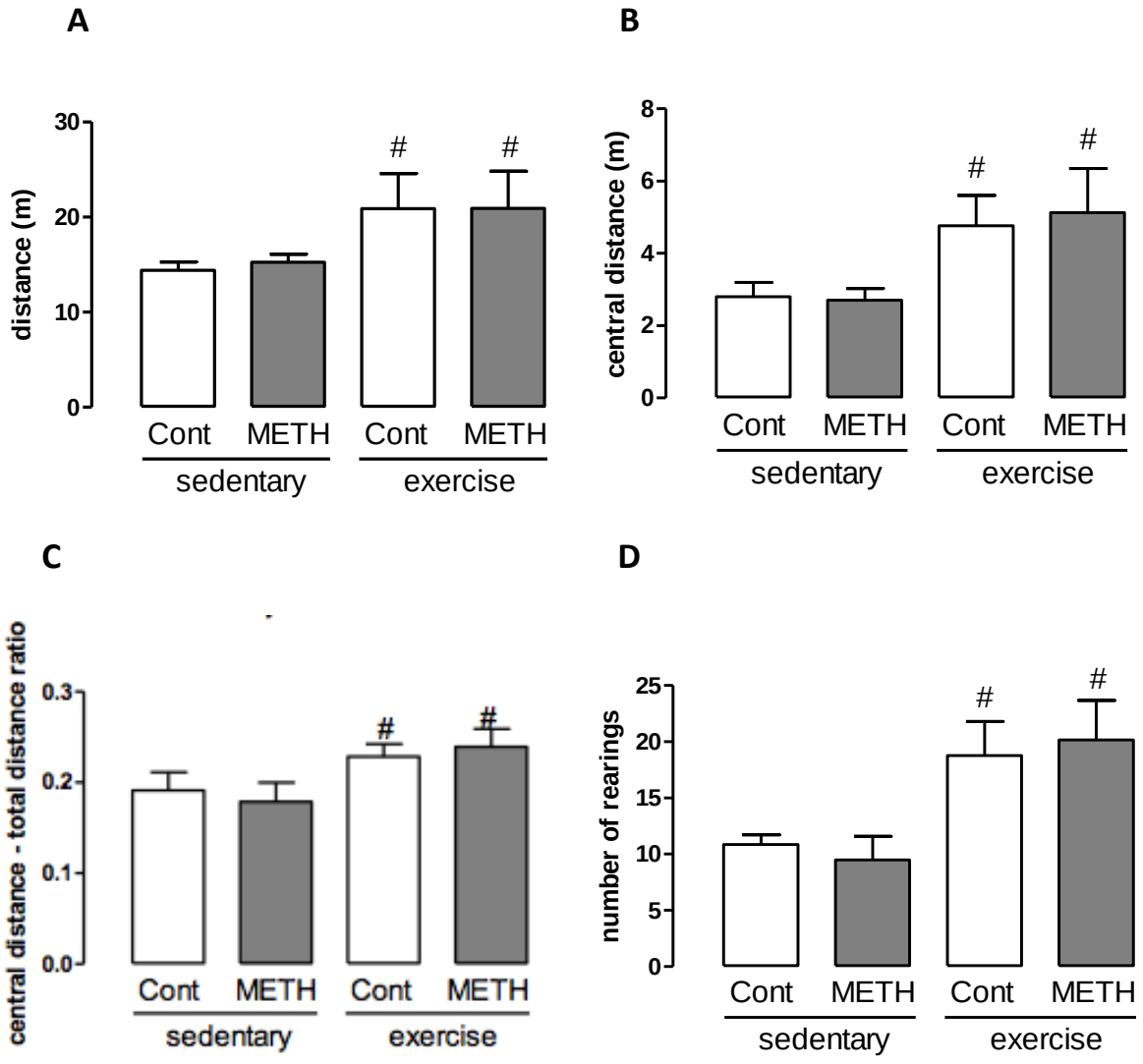



**Figure 18** – **A:** Evolution of the mice body-weight over 61 days (total duration of experimental protocol). **B:** Mice body-weight at 3 different time-points: day 1: beginning of training; day 14: METH administration; day 61: last day of physical exercise program. The results are expressed as mean ± S.E.M. of 6-8 animals per group. Control sedentary mice body-weight at the 61 days is statistically different from the other time points ($P<0.05). #P<0.05. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls multiple comparison.

# 2. Effect of treadmill exercise on general exploratory locomotion in METH-exposed mice

## 2.1. Open-field test

Mice activity was quantified in the open-field over 5 minutes to assess novel environment exploration 49 days post-METH injection. METH-sedentary mice showed normal total distance (Figure 19 A) and central distance travelled (Figure 19 B), normal central distance–total distance ratio (Figure 19 C), number or rearings (Figure 19 D) and average speed (Figure 19 E). This is suggestive that the general exploratory behavior of these animals is not statistically different from that showed by the respective controls (SAL/SED) ($P>0.05$). Exercise increased all these measured parameters in both METH and Cont ($P<0.05$). This is highly suggestive that physical activity enhanced both locomotor activity and exploratory behavior.



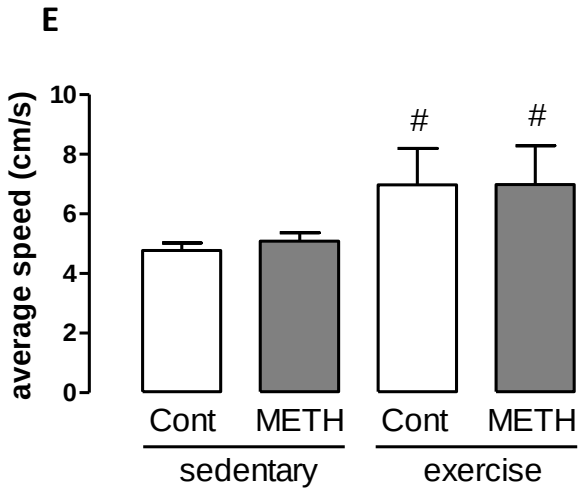



**Figure 19 –** Effect of treadmill exercise on the locomotor activity and exploratory behavior (open field test) in mice injected with a single dose of METH (30 mg/kg, i.p.). The figure shows the total and central area distances traveled (A,B), central distance-total distance ratio (C), number of rearings (D) and the average speed (D) in the arena at 49 days post-injection. The results are expressed as mean ± S.E.M. of 6-8 animals per group. #P<0.05 versus respective sedentary animal controls, using a two-way ANOVA with Bonferroni post-test.

# 3. Effect of treadmill exercise on depressive-like behavior in METH-exposed mice

## 3.1. Splash test

Mice anhedonic-like phenotype was assessed using the Splash test that allowed the measurement of grooming behavior for 5 minutes, 49 days post-injection.

A two-way ANOVA test disclosed a significant drug x exercise interaction ($F_{1,23}=5,92$, $P<0,05$) in grooming time. A post-hoc analysis showed that METH-sedentary mice exhibited reduced grooming time when compared with saline-sedentary. Notably, METH-exercised mice not only showed similar grooming time when compared with its exercised control ($P<0.05$) but also had higher grooming time when compared with METH-sedentary mice ($P<0.05$) (Figure 20 B). Although METH-sedentary mice showed a tendency to have an increased latency to grooming it did not attain significance when compared with their sedentary controls (Figure 20 A).

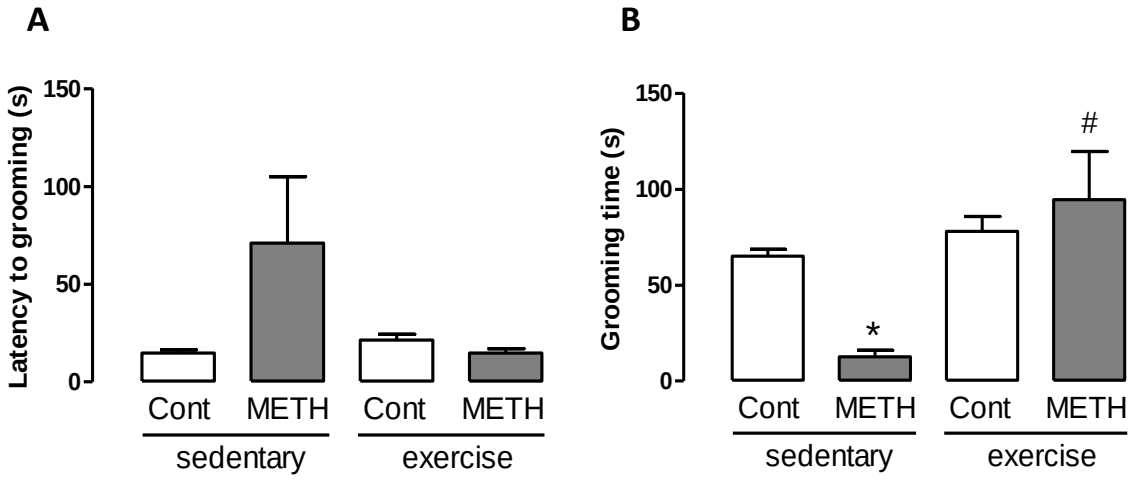



**Figure 20 –** Effect of treadmill exercise on grooming behavior (splash test) in mice injected with a single dose of METH (30 mg/kg, i.p.). The figure shows the latency to grooming (A) and grooming time (B) in the splash test at 49 days post-injection. The results are expressed as mean ± S.E.M. of 6-8 animals per group. *P<0.05 versus SAL/sedentary; #P<0.05 versus METH/sedentary, using a two-way ANOVA with Bonferroni post-test.

## 3.2. Forced-swim test

Mice despair-like behaviour was probed using forced swim-test for 5 minutes, 49 days post-METH injection.

METH-sedentary mice did not show a despair-like behavior as gauged by immobility, swimming and climbing times not being statistically different from those shown by their respective controls (SAL/SED) ($P>0.05$; Figure 21 A, B, C). Notably, treadmill exercise decreased the immobility time (Figure 21 A) and increased the swimming time (Figure 21 B) in both SAL and METH groups when compared to their sedentary controls ($P<0.05$; Figure 22 A, B, C). Nonetheless exercise failed to change the climbing time (C) relatively to sedentary groups ($P>0.05$; Figure 21 C).

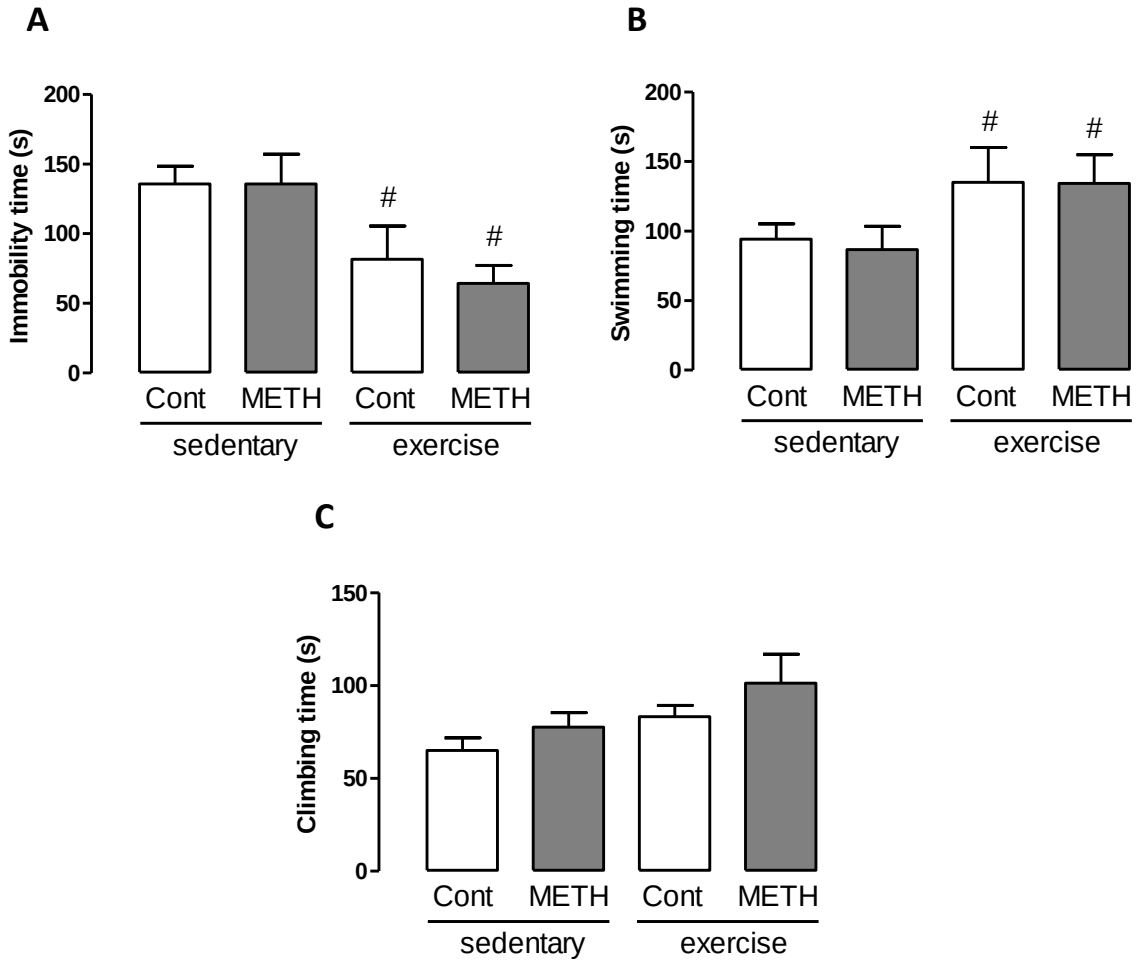



**Figure 21 –** Effect of treadmill exercise on the depair-like behavior (forced swimming test) in mice injected with a single dose of METH (30 mg/kg, i.p.). The figure shows the immobility (A), swimming (B) and climbing (C) times in the forced swimming test at 49 days post-injection. The results are expressed as mean ± S.E.M. of 6-8 animals per group. #$P<0.05$ versus respective sedentary control animals using a two-way ANOVA followed by with Bonferroni post-test.

## 4. Effect of treadmill exercise on 5-HT total content in frontal cortex and hippocampi from METH-exposed mice

Serotonin is a neurochemical fingerprint from depressive-like behavior and was measured herein by HPLC. Hippocampal 5-HT levels were quantified 3 and 49 days post-METH injection. Frontal cortical 5-HT levels were only determined at the final end-point. METH imposed a hippocampal 5-HT depletion as seen in Figure 22 A. However, neither drug nor exercise had a significant effect on cortical and hippocampal 5-HT total content (P>0.05; Figure 22 B,C).

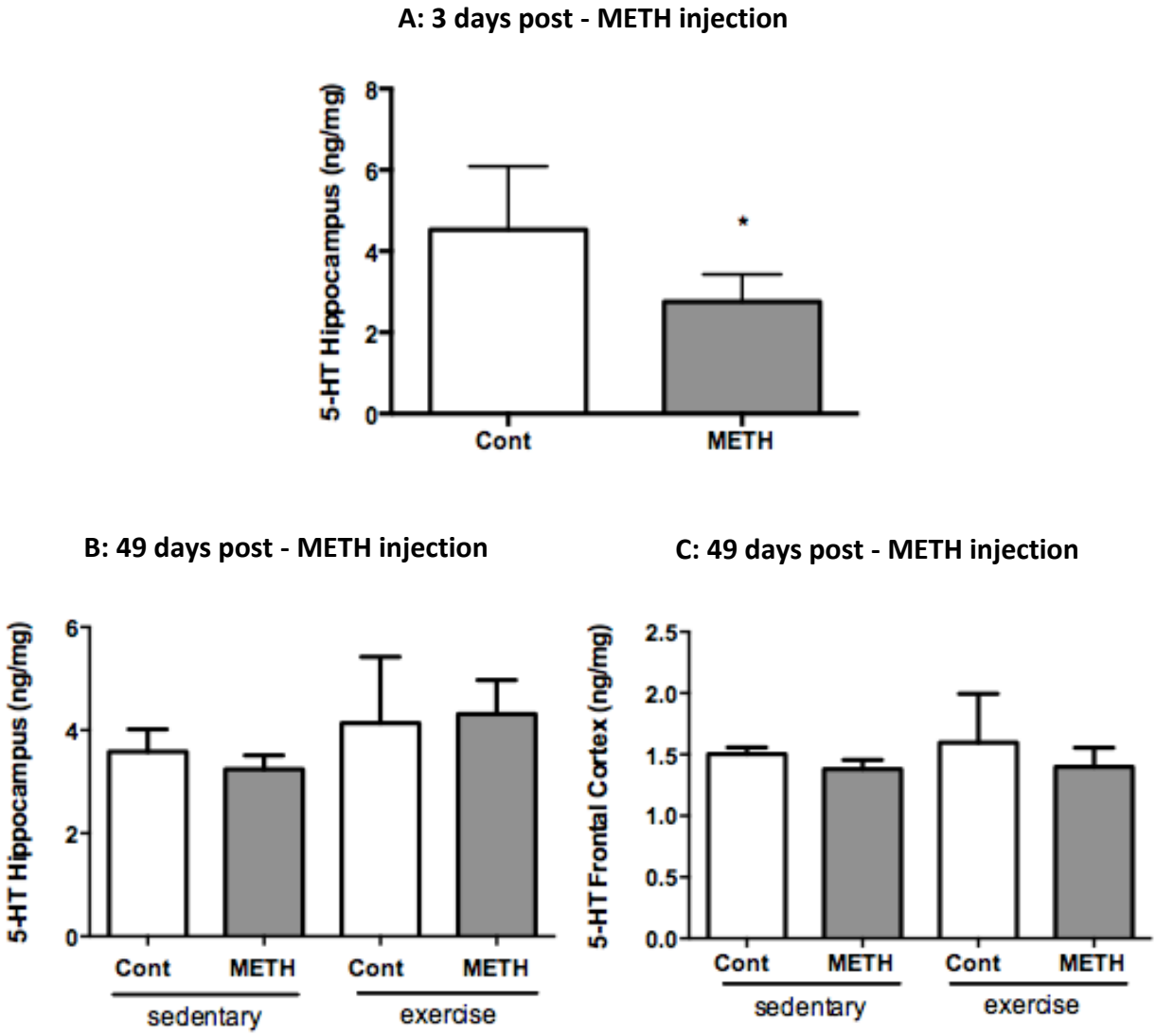



**Figure 22 –** Effect of treadmill exercise on hippocampal (A,B) and frontal cortical (C) serotoninlevels (5-HT measured from mice at 3 (A) and 49 days (B, C) post-METH injection). The results are expressed as mean ± S.E.M. of 6-8 animals per group. *P<0,05 versus saline-treated animals using unpaired t-test.

# CHAPTER 4

# DISCUSSION

# Discussion

Although clinical studies have shown the relevance of the symptoms of depression in consumers of METH on abstinence (Glasner-Edwards et al. 2009 and Volkow et al., 2001; Rusyniak, 2011), this psychiaytric abnormality in METH abuse context has been scarcely studied. In fact, the study of the long-term mood behavioural and underlying neurochemical consequences of METH consumption is still poorly characterized. Therefore herein we offer a comprehensive characterization of the long-term emotional state of mice following a single-neurotoxic dose of METH using Splash, forced swim test (FST) and open field (OF). We want ultimately to robustly confirm that METH induces depressive-like behaviour. We further aimed to establish a relationship between 5-HT homeostasis in frontal-cortex and hippocampus and the putative antidepressant effect induced by physical exercise.

The single high METH dose regimen that we use in the present study recapitulates the detrimental effects found in METH users including corticalstriatal dopaminergic and astroglial dysfunction (Cappon et al., 2000; Imam & Ali, 2001; Xu et al., 2005; Zhu et al., 2005; Krasnova & Cadet, 2009; O'Callaghan et al., 2008; Kitamura et al., 2010; Sailasuta et al., 2010; Pereira et al., 2012; Silva et al., 2014). In addition, this model reduces the inherent complexity present in repeated dosage regimens and reduces the occurrence of seizures and high mortality seen with multiple doses regimen (Davidson et al., 2001; Miller & O'Callaghan, 2003). In fact, in the present study, only two mice died and none showed convulsions or weight reductions. It is noteworthy to stress that animals from the exercise group started to put on weight following 14 days of adaptation to the treadmill. This might suggest that the animals had to cope with a stressful situation that they overcome as shown by weight evolution and behavioral phenotype (see above).

The splash test is a direct measure of self-care and motivational behavior and has been reported to probe for mood disorders in rodent models. We observed for the first time, that the METH regimen induced a decreased grooming behaviour in the METH-sedentary mice, when compared to the respective control, which is indicative of a long-term anhedonic-like behaviour that mimics apathy observed in clinical depression (Willner, 2005). Furthermore, METH-sedentary mice showed a tendency to had increased latency to grooming, further indicating loss of self-care and motivational behavior. This is consistent with the fact that some chronic METH users exhibit an anhedonic and

depressive behavior in a persistent manner after a few years follwoing the end of consumption (Rawson et al. 2002). Physical exercise, by itself, didn't alter the grooming time of the mice, but recovered grooming-time in METH-intoxicated mice. This result suggests that the practice of physical exercise is a non-pharmacologic strategy with anti-anhedonic potential (Willner et al., 2005). We further analysed negative mice behaviour using FST. This test was designed by Porsolt, as a primary screening test for antidepressants with a strong predictive validity (Porsolt et al., 1977). Porsolt et al. (1977) further proposed that the immobility time observed in this test reflected a state of lowered mood or hopelessness in animals (Cryan et al., 2002).

FST showed that METH-sedentary mice exhibited normal immobility, swimming and climbing times when compared with their sedentary controls. This is apparently contractidory with METH-intoxicated mice having increased immobility time in the TST at the same time-point (Silva et al., 2014). However, it was demonstrated that TST is more sensitive than FST in illustrating neurochemical abnormalities (St´eru et al., 1985; Castagn´e, et al., 2010; Chatterjee et al., 2011). On the other hand this might also further suggest that TST and FST might reflect different pathophysiological mechanisms underlying immobility as proposed by Chatterjee et al. (2011).

Finally, Castagn´e, et al. (2010) described numerous advantages of using TST over FST such as no induction of hypothermia and the animals once taken out from the experimental set up resume instantly normal spontaneous activity. More importantly, treadmill exercise decreased immobility time and increased swimming time, irrespectively of drug treatment, when compared to their sedentary controls. These behavioural observations clearly demonstrate that physical exercise is endowed with antidespair/antidepressant-like properties. This further corroborates our TST data showing that treadmill exercise decreased the immobility time spent by METH-intoxicated mice 49 days post-METH (Pereira et al., 2014, unpublished). These antidepressant-like properties are independent of locomotor behaviour activity since treadmill exercise had no effect on climbing time from both METH- and Cont- trated mice. We also evaluated novel environment exploration of mice 49 days post-METH injection using the OF test during 5 minutes. We demonstrated that METH regimen did not significantly alter the general physical motor abilities and level of interest in the novelty of the environment as gauged by similar total distance travelled, average speed and number of rearings compared to saline-treated animals, in the sedentary group. Interestingly, Timár et al. (2003) reported a decrease of spontaneous locomotor activity in rats 3 days after METH treatment (10

mg/kg, 4 subcutaneous injections every 2 hours). However, this effect was transient since 1, 2 and 4 weeks after METH administration, animals revealed a normal locomotor behavior. This further suggests that the anhedonic-like behavior showed by METH-intoxicated mice is independent of locomotor activity. However, chronic exercise increased all the measured OF parameters in both METH and Cont, suggesting that treadmill enhanced both locomotor activity and exploratory behavior. These results could be related with the high-mood state triggered by physical exercise as shown by both Splash and FST, irrespectively of drug treatment. Consistently, these mice exhibited an anxiolytic-like behaviour as gauged by increased center distance–total distance ratio compared to sedentary mice.

Articulating the three behavioral tests used herein, one can propose that treadmill exercise offers an antidepressant-like effect to mice submitted to a neurotoxic METH dose. This pre-clinical approach gives further credence for the reported benefitial effects of physical exercise to METH-abstinent individuals (Mooney et al., 2014).

We hypothesized that this beneficial impact of treadmill exercise is dependent upon its modulatory impact on 5-HT homeostasis in both hippocampus and frontal cortex from METH-intoxicated mice. Although METH imposed a robust hippocampal 5-HT depletion (*circa* 45%) at 3 days post-injection, this neurochemical detrimental effect was reversed 49 days post-injection. On the other hand, treadmill exercise failed to significantly change 5-HT levels in both saline and METH groups. Importantly, this means that physical exercise had no effect on 5-HT hippocampal terminal recovery in METH-intoxicated mice. Additionally, frontal cortical 5-HT levels from METH sedentary-intoxicated mice were not statistically different from their control saline mice. On the other and, Silva et al. (2014) showed that, METH imposed a 5-HT depletion of circa 25% in frontal cortex. Although apparently contradictory, these results stress that the 5-HT depletion on frontal cortex was not robust enough to be reproducible. One can also add that this METH regimen is more detrimental to dopaminergic systems compared to serotonergic systems (Pereira et al., 2012; Silva et al., 2014). Consistently with hippocampal 5-HT data, treadmill exercise failed to significantly change 5-HT levels in frontal cortex from both saline and METH groups. Overall, one can discard the hypothesis that treadmill exercise exerted an antidepressant-like effect through modulating 5-HT synthesis in both of hippocampal and frontal-cortex. However, one cannot disregard the hypothesis that METH-intoxicated mice had changes in 5-HT extracellular basal levels and signaling. This warrants further scrutiny.

Disruption in the neurotrophic factors, which play a major role role in neuroplasticity, could be also held responsible for the depressive-like behavior METH-induced (Matta et al., 2013). Interestingly, it was also postulated that a reduction of neurotrophic factors in limbic structures, including the hippocampus and prefrontal cortex is directly involved in the pathophysiology of depressive disorders (Duman & Monteggia, 2006). Therefore, antidepressants might mediate their therapeutic benefit, in part, by increasing levels of these factors in the hippocampus.

Importantly, it was shown that serum brain-derived neurotrophic factor (BDNF) levels were significantly downregulated in METH abusers during the first 3 weeks of withdrawal (Chen et al., 2012). Another study found that chronic METH abusers who had been abstinent for at least 1 month had significantly higher plasma BDNF levels compared to healthy control subjects (Kim et al., 2005).

Taken together, these studies strongly suggest that BDNF, was shown to be decreased first and then increased after METH withdrawal in humans. These findings are compatible with findings from animal studies. For example, neurotoxic-METH treatment in rat increased BDNF in multiple brain regions from rat including frontal cortex and hippocampal CA1 (Braun et al., 2011). Therefore probing the role of BDNF in the beneficial treadmill exercise in METH-intoxicated mice is warranted.

On the other hand, there is also substantial evidence showing that regular physical exercise is associated with a low reactivity of the sympathetic nervous system (SNS) and the HPA axis, that plays a critical role in developing responses to psychological and physical stressors (Stranahan et al., 2008 and Rimmele et al., 2009). Specifically, physically active subjects showed lower cortisol increase (Rimmele et al., 2007), to psychological laboratory stressors in comparison with less active controls. Thus, exercise-induced changes in HPA axis might modulate negative affects in humans. Importantly, adolescent METH use is associated with greater psychiatric symptoms and enhanced cortisol secretion following a social stressor, particularly in younger female METH users (King et al. 2010). Therefore the relevance of the HPA axis to the antidepressive-like effect of treadmill exercise also warrants further analysis. Additionally other brain regions including amygdala should be scrutinized.

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# CHAPTER 5

# CONCLUSION

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# Conclusion

This study is relevant because it further demonstrated that a single-high METH dose imposes a long-lasting depressive-like symptoms such as anhedonia. Furthermore, we unequivocally show that physical exercise is a non-pharmacologic strategy that offers antidepressive-like effects to METH-intoxicated mice. We also demonstrated that this treadmill effect was independent of 5-HT homeostasis in both frontal-cortex and hippocampus. Therefore, further studies are warranted to understand the neurochemical basis for this positive behavioral effect.

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# CHAPTER 6

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