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Hugo Rafael Santos Ferreira

CHARACTERIZATION OF THE EMOTIONAL FINGERPRINT OF METH INTOXICATED ANIMALS

Dissertação no âmbito do Mestrado em Química Medicinal,
orientada pelo Professor Doutor Frederico Guilherme de Sousa da Costa Pereira,
e apresentada ao Departamento de Química da Faculdade de Ciências e Tecnologia
da Universidade de Coimbra.

Setembro de 2019

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Resumo

A saúde mental deve ser vista como uma fonte de capital humano e bem-estar na sociedade (WHO, 2019). Doenças mentais são o reflexo de perturbações na saúde mental de um indivíduo que são expressas através de uma combinação de pensamentos problemáticos, labilidade emocional, alterações comportamentais e nas relações interpessoais. Transtornos depressivos e ansiosos são os transtornos mentais mais comuns a nível mundial (World Health Organization, 2017a). Estas doenças mentais estão muitas vezes relacionadas com o abuso de substâncias (incluindo o uso recreacional de metanfetamina) e tornaram-se um assunto relevante nas políticas de drogas e no fornecimento de tratamentos (Zweben *et al.*, 2004).

A atividade física tem sido documentada como tendo um efeito nos transtornos mentais incluindo os transtornos ansiosos e depressivos (Dishman *et al.*, 2006; Phillips, 2017a).Os tratamentos atuais para estes transtornos não têm demonstrado a eficácia desejada na maior parte dos casos (Peluso and Andrade, 2005; Ren, Luan, Zhang, Gutteea, Cai and Zhao, 2017).

Este estudo tem como objetivo encontrar e caracterizar a *emotional fingerprint* do uso indevido de metanfetamina, nomeadamente anxiety *and depressive-like behaviors,* e o impacto do exercício físico em murganhos intoxicados com metanfetamina. Um protocolo de administração de metanfetamina foi usado para induzir os *anxiety and depressive-like behaviors* e após foi posto em prática um protocolo de exercício físico como estratégia de correção dos comportamentos.

Este estudo não conseguiu gerar nenhum efeito negativo visível no estado emocional dos animais intoxicados com metanfetamina. Adicionalmente o protocolo de exercício físico não gerou diferenças locomotoras nem emocionais nos animais exercitados. Sendo assim, é necessária mais investigação para caracterizar os sintomas ansiosos e depressivos encontrados nos toxicodependentes em fase de abstinência.

PALAVRAS-CHAVE: transtornos do humor, metanfetamina, exercício físico, murganhos, análise comportamental.

Abstract

Mental health should be seen as a valued source of human capital or well-being in society (WHO, 2019). Mental disorders represent disturbances to a person's mental health that are often characterized by some combination of troubled thoughts, emotions, behavior and relationships with others. Depressive and anxiety disorders are the most common mental disorders (World Health Organization, 2017a). The presence of these mental disorders are associated with drug abuse (including methamphetamine misuse) and become an important issue in drug policy and treatment provision. (Zweben et al., 2004).

Physical activity has been documented to have a role on mental health, including in anxiety and depression (Dishman *et al.*, 2006; Phillips, 2017b).

The effectiveness of current available treatments for these psychiatric disorders is often modest (Peluso and Andrade, 2005; Ren, Luan, Zhang, Gutteea, Cai and Zhao, 2017).

This study is aimed at finding the emotional fingerprint of methamphetamine misuse, namely anxiety and depressive-like behavior, and the impact of physical activity in mice intoxicated with methamphetamine. A methamphetamine administration protocol was used to induce depressive-like and anxiety-like behaviors in mice and a physical exercise protocol was employed as an ameliorative strategy.

We failed to see any negative impact of METH on mice emotional status. Additionally, physical exercise did not significantly change locomotor and emotional parameters in mice. Therefore, more research is needed to further characterize the well-known depressive and anxiety symptoms seen in METH addicts undergoing withdrawal.

KEYWORDS: Mood disorders, Methamphetamine, physical exercise, mice, behavioral analysis.

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1. Introduction

1.1. Common mental disorders

Mental disorders are generally characterized by a combination of abnormal thoughts, perceptions, emotions, behavior and relationships with others. The burden of mental disorders continues to grow with significant impacts on health and major social, human rights and economic consequences in all countries of the world (Leray, 2018). Common mental disorders refer to two main diagnostic categories: depressive disorders and anxiety disorders. In fact, these disorders are highly prevalent in the population and impact on the mood or feelings of affected persons (WHO, 2017)

1.2. Depressive disorders

Depressive disorders are a very serious mood disorder. There are a lot of different depressive disorders such as, major depressive disorder (MDD) commonly referred to as depression , persistent depressive disorder (PDD) also known as dysthymia and substance/medication abuse induced depressive disorder (APA, 2014).

An individual can be diagnosed with MDD when it has at least five specific symptoms present for at least two weeks including but not limited to depressed

mood, angry outbursts, weight changes, fatigue, low self-worth or inappropriate guilt, decreased concentration and recurrent thoughts of death and suicide (Bentley, Pagalilauan and Simpson, 2014).

In 2015 it was estimated that about 4,4% of the world population had depression meaning 322 million people lived with depression. Depression has different prevalence in males (3,6%) and females (5,1%) and is also influenced by age peaking in the older adulthood, in the 55-74 age interval. Depressive disorders are ranked as the number one contributor to non-fatal health loss worldwide and in 2015 people who suffered from depression accounted to a total of 50 million YLD, as shown in Table 1 (World Health Organization, 2017b).

Psychological, physiological and social factors can augment the risk of developing depression. For example, chronic pain, abuse of prescribed or illicit drugs, incurable diseases, being fired from their job, incarceration and low educational level (APA, 2014; Loula and Monteiro, 2019). Moreover, depressive disorders show some comorbidity with substance use disorder (Bentley, Pagalilauan and Simpson, 2014).

Finally, there exists a large body of epidemiological prospective data showing that people with severe mental illness, including major depressive disorder have an increased risk of developing coronary heart disease (CHD), compared with controls.

Several studies have shown a high comorbidity between MDD and metabolic syndrome (a cluster of obesity, insulin resistance, hypertension, impaired glucose tolerance or diabetes, hyperinsulinemia, elevated triglycerides and low high-density lipoprotein [HDL] concentrations) (Eckel *et al.*, 2010; Pan *et al.*, 2012; Rethorst, Bernstein and Trivedi, 2014; Salvi *et al.*, 2017; Gramaglia *et al.*, 2018). Moreover, there seems to be an association among depressive symptoms, antidepressants therapy and metabolic syndrome (Gramaglia *et al.*, 2018)

Psychotherapy is one of the possible interventions for MDD and it can be divided into CBT, behavioral activation therapy and psychodynamic therapy. In a 2014 meta-analysis showed that 62% of patients who underwent psychotherapy were no longer showing signs of MDD, compared to the improvement of traditional care (48%) (Bentley, Pagalilauan and Simpson, 2014; Huhn *et al.*, 2014). Another

way to intervene in MDD is using a pharmacotherapy approach with antidepressants, which include multiple categories namely selective serotonin reuptake inhibitors (SSRIs; e.g. fluoxetine), serotonin-norepinephrine reuptake inhibitors (SNRIs; e.g. venlafaxine), serotonin modulators and stimulators (SMSs; e.g. vortioxetine), tricyclic antidepressants (TCAs; e.g. amitriptyline), noradrenergic and specific serotonergic antidepressant (NaSSA; e.g. mirtazapine, norepinephrine-dopamine reuptake inhibitor (NDRI, bupropion) and monoamine oxidase inhibitors (MAOIs; e.g. moclobemide) (Gartlehner *et al.*, 2008).

Depressive disorders	Total YLD (thousands)	YLD per 100,000	% of all YLDs	Rank cause
Low- and middle-income countries				
- African Region	7229	731	7.9	2
- Eastern Mediterranean Region	4049	685	6.9	2
- European Region	3517	859	8.1	2
- Region of the Americas	5106	844	9.3	1
- South-East Asia Region	13967	724	7.0	2
- Western Pacific Region	10525	640	7.2	2
High-income countries	9608	839	7.9	2
World	54215	738	7.5	1

Table 1 - Global and regional estimates of Health Loss due to depressive disorders. Table retrieved from World Health Organization, 2017b.

1.3. Anxiety Disorders

At some point in life, everyone faces situations that make them feel fear or anxiety, and although these are normal emotions to feel they sometimes are a symptom of an anxiety disorder. Fear is an emotional response to an imminent threat, real or perceived, and anxiety is the anticipation of a future threat. Anxiety disorders differ from the adaptative fear and anxiety in duration, intensity, and frequency.

Anxiety disorders	Total YLD (thousands)	YLD per 100,000	% of all YLDs	Rank cause
Low- and middle-income countries				
- African Region	2639	267	2.9	7
- Eastern Mediterranean Region	2093	354	3.6	7
- European Region	1239	302	2.9	8
- Region of the Americas	3433	567	6.2	3
- South-East Asia Region	5522	286	2.8	9
- Western Pacific Region	4506	274	3.1	8
High-income countries	**5061**	**442**	**4.2**	**4**
World	**24621**	**335**	**3.4**	**6**

Table *2* – Global and regional estimates of Health Loss due to anxiety disorders. Table retrieved from World Health Organization, 2017b.

There are various types of anxiety disorders, such as generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), Social Anxiety Disorder (SAD), agoraphobia, panic disorder (PD), post-traumatic stress disorder (PTSD) and substance/medication induced anxiety disorder (APA, 2014).

According to a Global Health Estimates report, the global population that suffers from anxiety disorders was estimated to be 3,6% as of the year 2015, with a 2:1 ratio regarding female *vs* male prevalence. Also, anxiety disorders are ranked as the sixth largest contributor to non-fatal health loss worldwide and its sufferers accumulated a total of 24,6 million of Years Lived with Disability (YLD), as shown in Table 2 (World Health Organization, 2017b).

Anxiety disorders often have comorbidity with depressive disorders or substance abuse disorders (Costello, Egger and Angold, 2005). Female gender, disturbed family environment, low self-esteem and lower educational attainment were shown to increase the risk of developing any anxiety disorder (Blanco *et al.*, 2014). People suffering from anxiety disorders are at higher risk of cardiometabolic diseases, such as diabetes and acute cardiac events (Edmondson & von Kanel, 2017; Smith, Deschenes, & Schmitz, 2018). This is potentially due to shared etiological biological factors between anxiety and cardiovascular disorders (e.g., increased inflammation and oxidative stress; Belem da Silva et al., 2017), but also

due to modifiable risk behaviors like lower physical activity (PA) levels and increased sedentary behavior (Stubbs, Koyanagi et al., 2017; Vancampfort, Stubbs, Herring, Hallgren, & Koyanagi, 2018).

Anxiety disorders can be managed with cognitive behavioral therapy (CBT) (Carpenter *et al.*, 2018) and pharmacological agents such as benzodiazepines and antidepressants including selective serotonin reuptake inhibitors (SSRIs; e.g. sertraline) and serotonin-norepinephrine reuptake inhibitors (SNRIs) (Baldwin *et al.*, 2005; Baldwin, Waldman and Allgulander, 2011) or a combination of both pharmacological agents and CBT (Ori *et al.*, 2015). Moreover, physical exercise (PE) has also a role in managing anxiety disorders (Kandola *et al.*, 2018).

1.4. Methamphetamine addiction

Drug addiction is a chronic, relapsing mental disorder in which a person develops a compulsion to consume or use a drug despite some severe negative consequences. Drug addiction encompasses a harmful pattern of use of addictive substances as follows: occasional use, recreational use, regular use and addiction. Furthermore, the social context and the person's genetic and psychological structure play a vital role in the process of becoming addicted (Nestler, 2001; Koob, 2017).

In order to understand addiction and its mechanisms it was mandatory to understand and characterize drugs of abuse targets. Indeed, it is now well-established that, for instance, opiates act as agonists of opioid receptors, cocaine and amphetamines are indirect agonists of dopamine receptors (even though their mechanism of action differs), nicotine is an agonist of nicotinic acetylcholine receptors, hallucinogens are partial agonists of serotonin receptors, cannabinoids are agonists of the cannabinoid receptors and ethanol interacts with the $GABA_A$ (gamma-aminobutyric acid) and NMDA (N-methyl D-aspartate) receptors (Nestler, 2001). However, the rewarding properties of nearly all drugs of abuse is associated with their impact in the mesocorticostriatal dopamine systems (Koob and Volkow, 2016).

Drug addiction has been hypothesized to be composed of a three-stage recurring cycle that worsens over time: 1) binge/intoxication, 2) withdrawal/negative effect and 3) preoccupation/anticipation. These 3 stages are characterized by disturbances in the following three major neurocircuits 1) basal ganglia; 2) extended amygdala; 3) prefrontal cortex, respectively.

In the context of drug addiction, individuals move from impulsivity to compulsivity, and the drive for drug-taking behavior is paralleled by shifts from positive to negative reinforcement (Figure 1). The positive reinforcement is a process in which there's an increase of the probability of response when presented a stimulus and the negative reinforcement process is defined by an increase in probability of response when an aversive stimulus is removed (Figure 1) (Koob and Le Moal, 2008; Koob, 2017).

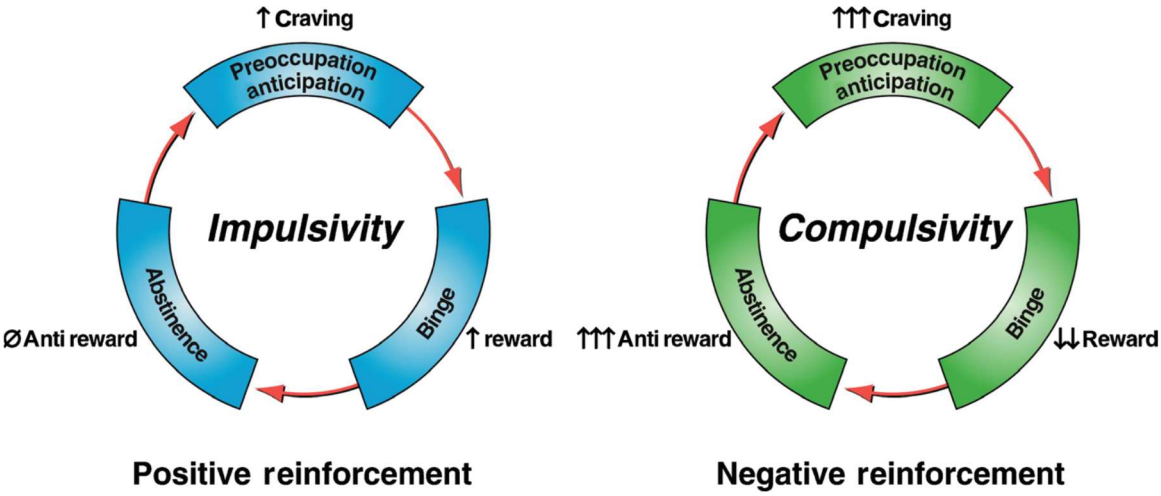


Figure 1 – Addiction cycle and its 3 major components. Image retrieved from Koob and Le Moal, 2008.

In 2016 about 275 million people worldwide between 15-64 years used drugs at least once, 34 million of those used amphetamines and prescription stimulants. The number one drug that causes the most harm to its consumers is the opioid class, which includes heroin, followed by the amphetamines, with methamphetamine (METH) as its most harmful type (United Nations Office on Drugs and Crime, 2017; United Nations Office of Drugs and Crime, 2018).

Amphetamines and amphetamine type drugs when consumed cause a mood enhancement, a state of euphoria as well as an increase in alertness, a decrease in fatigue felt and social and sexual disinhibition (Scott *et al.*, 2007; Kitanaka *et al.*, 2010; Cunha-Oliveira *et al.*, 2013). Amphetamines are currently

used in the treatment of narcolepsy, ADHD but are also used as recreational drugs, mostly methamphetamine because it is more potent than its parent compound (Figure 2).

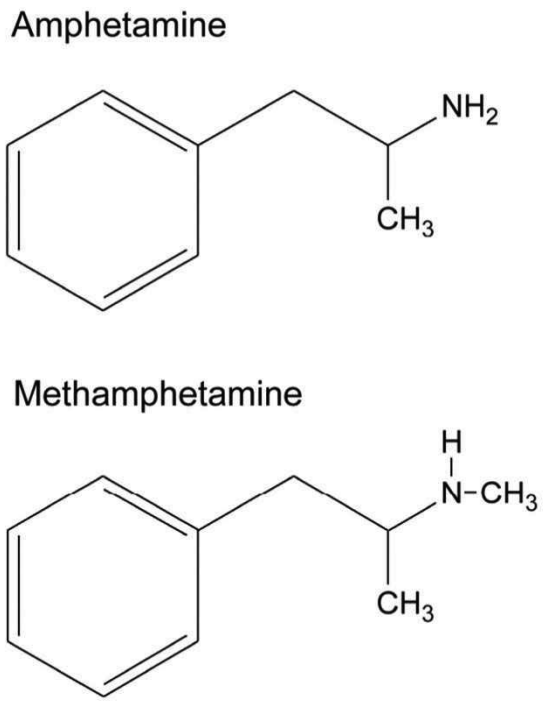


Figure 2: Comparison of chemical structures of methamphetamine and amphetamine. Methamphetamine differs from its metabolite amphetamine by the presence of a methyl group. Both produce the same stimulant behavioral effects. Image retrieved from Kish, 2008.

METH has been called as an epidemic drug in the United States of America as it's a known drug of choice to party-goers, namely in raves, and also an energy booster for athletes, soldiers and long-distance truck drivers (Cunha-Oliveira *et al.*, 2013).

Given that METH is highly lipid soluble and is small-sized it can easily cross the blood brain barrier (BBB). Once it permeates the BBB, METH increases the extracellular levels of dopamine (DA) through the following mechanisms: 1-redistribution of DA from synaptic vesicles (via the vesicular monoamine transporter VMAT2) to the neuronal cytoplasm and 2-the reverse transport of neurotransmitters through the plasma membrane transporter into the extracellular space (Figure 3) (Kish, 2008). Other monoamine neurotransmitters (serotonin, norepinephrine) are also released by METH. METH is usually consumed orally, intranasally, intravenously administered or it can be smoked (Kish, 2008).

Prolonged METH consumption is related to the development of psychiatric disorders including depression and anxiety (please see section 1.7) (London *et al.*, 2004).

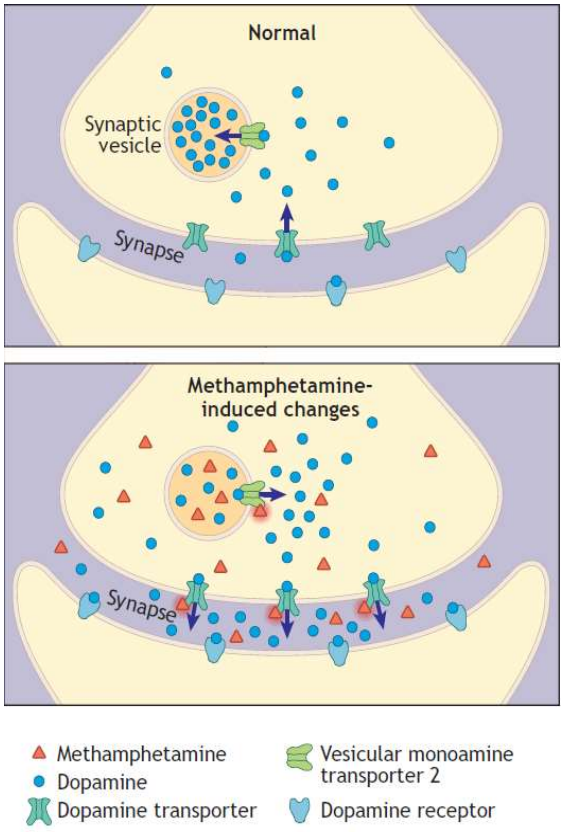


Figure 3 How methamphetamine targets synapses and changes the extracellular levels of dopamine. Image retrieved from Kish, 2008.

1.5. Physical Exercise and its benefits in general health

Although physical exercise (PE) and physical activity have been interchangeably used and share some common elements, they're not the same thing (Caspersen, Powell and Christenson, 1985). Physical activity results of skeletal muscles contraction and produces body movement thus leading to energy expenditure. Whether a person is doing household chores like yard work, house cleaning, dish-washing or even simply walking down the street is engaging in physical activity. The energy expenditure is usually measured in kilocalories(kcal), but it can also be measured in kilojoules(kJ). Physical exercise is a specific type of physical activity that revolves around the maintenance or improvement of a person's physical fitness, including mental health. Physical activity becomes physical exercise when it's structured, repetitive and planned .

There are two types of PE, the aerobic and the anaerobic exercise. The first is defined as the type of exercise that occurs while the cardiovascular system can supply oxygen to the skeletal muscles involved in the exercise (Patel *et al.*, 2017). To access the aerobic capacity of an individual it's necessary to measure or calculate the peak of oxygen consumption (VO_{2max}). This is normally achieved via treadmill protocols coupled with an oxygen consumption analyzer or via mathematical formulas. Jogging, swimming, dancing and walking are all examples of aerobic exercise.

Anaerobic exercise, contrary to the aerobic exercise doesn't rely in the use of oxygen to provide energy for the skeletal muscles involved in the exercise. Anaerobic exercise relies in the energy sources within the muscles contracting and are independent of the oxygen inhaled (Ferguson, 2014). This type of exercise is less energy efficient but the obtention of ATP (adenosine triphosphate) is much faster. High-intensity interval training (HIIT), powerlifting and sprinting are good examples of anaerobic exercise.

In aerobic metabolism the cells obtain ATP from carbohydrates and fatty acids with the help of O_2 inhaled, getting a lot of energy but this process takes time to obtain ATP (Ferguson, 2014; Patel *et al.*, 2017). When in anaerobic metabolism cells retrieve energy from glycolysis and fermentation forming lactic acid in the

process. This type of metabolism generates lesser ATP than the aerobic metabolism and the formation of lactic acid usually leads to muscle pain post-train.

Physical exercise has been more and more frequently associated with a healthier lifestyle. Regular physical activity has been proven to reduce the risk of developing chronic diseases(Chakravarty, 2008). In fact, regular practice of physical exercise has been well-established as a non-pharmacological strategy aiming to prevent and manage cardiometabolic diseases, including type 2 diabetes mellitus (Blatt and Gostic, 2018; Strollo *et al.*, 2019). Additionally, PE can prevent falls and promote independent living in older adults (Bull *et al.*, 2017), and prevent osteoporosis by generating and maintaining peak bone mass (Bielemann, Martinez-Mesa and Gigante, 2013; Kemmler *et al.*, 2015). Physical exercise is also a mean to achieve a longer life-span (Katzmarzyk, Gledhill and Shephard, 2000; Ferguson, 2014; Patel et al., 2017; Safdar and Tarnopolsky, 2017). Other health and social benefits stem from the positive impact of PE in brain, such as preventing and treating depression and anxiety (Warburton, Nicol and Bredin, 2006), preventing cognitive decline and dementia (Scholz *et al.*, 2009) (please see section 1.6).

Although the exact mechanisms remain uncertain, one can assert being physically active during its lifetime is beneficial at almost all levels. For example, practicing exercise leads to a decrease in immunosuppression, hypertension, oxidative stress and an increase in angiogenesis and neurogenesis and mitigation of peripheral sympathetic activity (Dishman *et al.*, 2006; Rockwood and Middleton, 2007; Bridle *et al.*, 2012; Carayol *et al.*, 2013; Patel *et al.*, 2017; Phillips, 2017a).

On the other tip of the scale resides physical inactivity which has proven to be a potential burden to the public health as it increases the risk of life changing chronic diseases for instance type 2 diabetes mellitus, coronary artery disease and osteoporosis (Katzmarzyk, Gledhill and Shephard, 2000; Dishman *et al.*, 2006).

1.6. Physical exercise, the brain, mood and anxiety improvements

As the world's population of elderly people continues to grow, mental health is becoming a bigger concern (Phillips, 2017a).

Practicing PE in a chronic manner improves brain health, as it improves the quality of sleep, cognitive functions and neuroplasticity in young to older adults and even in the elderly (Dishman *et al.*, 2006; Phillips, 2017b). Importantly, aerobic exercise emerged as an effective antidepressant intervention (Morres *et al.*, 2019).

In addition, a growing number of studies have shown that PE protects against the development of anxiety and is useful as an anxiety treatment (Schuch *et al.*, 2019)

The regular practice of exercise has been proven to promote the synthesis and release of neurotransmitters (including monoamines), endogenous opioids (namely beta-endorphins) and neurotrophic factors, as well as to increase neurogenesis and angiogenesis, thus affecting the brain (Peluso and Andrade, 2005). On the other hand, one of the proposed mood improvement mechanisms postulates that when engaging in physical exercise it is challenging and therefore gets the athletes immersed in the activity thus improving the mood (Duclos, Gouarne and Bonnemaison, 2003; Droste *et al.*, 2007). Another hypothesis is the distraction factor that suggests that when practicing physical exercise, the attention of the athlete diverts from any negative stimuli during and after exercising, hence improving the mood (Morgan, 1985; Peluso and Andrade, 2005)

It has been postulated that the aerobic exercise that creates a better affective response and adherence to training regimens is located near the 65% VO_{2max} turning the exercise more pleasurable and ensuring a more frequent practice (Matta Mello Portugal *et al.*, 2013).

1.7. METH withdrawal, anxiety, depression and physical exercise

After repeated used of METH, its positive effects start to become less and less pleasurable and consumers experience the onset of the negative reinforcement phase, in which they start to feel bad when not binging and start craving METH to reduce the unpleasant feeling of not being high on METH (Koob and Le Moal, 2008; Kitanaka *et al.*, 2010). In fact, within the early withdrawal phase most METH addicts are known to have strong craving feelings that are very persistent and intrusive, very often leading to relapses (Su *et al.*, 2017; Luan *et al.*, 2018). Moreover, METH addicts showed signs of anhedonia, anxiety, social inhibition, agitation, hypersomnia and paranoia when in the withdrawal phase (Zweben *et al.*, 2004).

Regarding preclinical studies, Kitanaka *et al.*, 2010 using a 10-day protocol of METH administration (1 or 2,5 mg/kg depending on the experimental group) followed by a 5-day withdrawal phase showed that the mice acquired an anxiety related behavior in the elevated plus maze, showing a decrease of time spent in open arms. The result showed a correlation with the expected human behavior for METH withdrawal (Zweben *et al.*, 2004; Scott *et al.*, 2007).

Using wistar rats Miladi-Gorji, Fadaei and Bigdeli, 2015 , and exposing them to a 2mg/kg dose of METH daily for 14 days followed with a 14 day withdrawal phase showed that the rats developed an anxiety like behavior in the elevated plus maze test.

Ru *et al.*, 2019 used C57BL/6 mice to experiment using an 8 weeks (5 days per week) of METH (5mg/kg) administration followed by a 1 week period of withdrawal to access the depressive/anxiety like behavior in the METH intoxicated mice. Several behavior tests were performed such as, forced swim test, sucrose preference test and open field test to detect the possible changes in the emotional status of the mice. The results showed that in the forced swim and the sucrose preference tests mice showed a depressive like behavior and in the open field test was possible to observe an anxiety like behavior in the mice. METH caused no locomotor changes.

Emotional alterations associated with METH may reflect neurochemical (including monoamine and neurotrophic factors) and metabolic impairments as suggested by preclinical and clinical studies (Berman *et al.*, 2008; Ren, Luan, Zhang, Gutteea, Cai, Zhao, *et al.*, 2017; Thanos *et al.*, 2017)

There's no current pharmacological cure to METH withdrawal, and the pharmacological and psychological approaches to deal with the withdrawal related syndromes have limited efficacy. Seeking help through medication or therapy is not in the range of everyone so its access is hard. On top of that anxiolytics and antidepressants show a far from desirable effect and psychotherapy suffers from the same problem, due to the lack of adherence to the treatments.(Peluso and Andrade, 2005; Larun *et al.*, 2006; Krogh *et al.*, 2017; Ren, Luan, Zhang, Gutteea, Cai, Zhao, *et al.*, 2017).

Physical exercise has been postulated to help prevent addictive behaviors, to attenuate symptoms of depression and anxiety and as aforementioned improve sleep quality and decreasing cognitive impairments. All of these features are very concurrent in METH users. Zschucke, Gaudlitz and Ströhle, 2013 showed preliminary results that pointed to a potential ameliorative effect of physical exercise/physical activity in physical and subjective aspects of mental disorders.

Morais *et al.*, (2018) presented evidence that a physical exercise protocol could decrease the anxiety and depression symptoms of METH addicted subjects. For example, Rawson *et al.*, 2015 by separating 135 METH users into two different groups, one receiving a 3-times-per-week 60 minute exercise protocol, during 8 weeks and the other receiving an equivalent time of health education sessions, demonstrated that the exercise group had substantially decreased their depressive and anxiety scores measured by the Beck Depression Inventory and Beck Anxiety Inventory, relatively to the health education group.

Wang *et al.*, 2017, showed that the craving and the inhibitory behavior of METH users could be targeted with the introduction of an aerobic exercise protocol (using a 12 week program in which the participants would jog or cycle, maintaining a 65-75% capacity of the participants' maximum heart rate), lowering both the levels of craving and an increase in the inhibitory control behavior.

However, the role of exercise in modulating the emotional behavior and its potential to be an adjuvant type of treatment in mood/anxiety disorders is not yet well established.

2. Objective

This study has two aims, to characterize the METH emotional fingerprint and to further demonstrate that physical exercise may correct mice emotional status.

3. Material and methods

3.1. Animals

56 C57BL/6J male mice were used in this project. The age of the animals was 8-10 weeks old, corresponding to earlier adulthood, and the weight of the animals ranged from 20,5 to 29,4 g, as of the start of the experiment. The animals were provided by the vivarium of the Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine of the University of Coimbra (FMUC).

The mice were accommodated in cages in groups of 1 to 3 according to brooding and were kept in the iCBR vivarium extension at the Institute of Pharmacology and Experimental Therapeutics/FMUC. The environment settings were as follows: temperature 22 ± 1 ºC, humidity 50 ± 10% and 12:12 h light-dark cycle (7 a.m. to 7 p.m.). Water and food were supplied *ad libitum*. As there was physical activity involved in some protocols the chow given to the animals was 4RF25, which has a higher protein content than the normal chow provided in the vivarium. The food and water consumption were weekly measured as well as the mice's weight to keep track of physiological evolution and to use as a marker for possible differences between experimental groups.

All the experimental procedures were properly done in accordance to the Technical Standards for the Protection of Animals User for Experimental and Other Scientific Purposes (Ordinance nº 129/92, of 6 July), laid out by the European Convention of Animal Welfare (Ordinance nº 1005/92). All experimental work was performed accordingly to the European Community guidelines (2010/63/EU). The number of animals used, and the suffering caused was minimized to the smallest amount possible.

3.2. Experimental design

This project considered 4 experimental approaches. Particularly, animals from Lot 1 (n=8), 3 (n=8) and 4 (n=8) were subjected to a single methamphetamine (METH) administration (30mg/kg, i.p.) and then subjected to an 8-week treadmill protocol (Experimental protocol I); mice from Lot 2 (n=8) were administered with a single METH administration (30 mg/kg i.p.) and left in the vivarium during 8 weeks (Experimental protocol II), Lot 5 (n=8) was administrated with 3 daily doses of METH (30 mg/kg i.p.) and left in the vivarium for 2 weeks (Experimental protocol III), and Lots 6 (n=7) and 7 (n=7) (Experimental protocol IV)were administered a daily dosage of METH (5 mg/kg i.p.) in the first week of the protocol and in the second week of the protocol another daily METH (10 mg/kg i.p.) administration and then left in the vivarium for the following 3 weeks. All the animals were subjected to a battery of locomotor and emotional evaluation. Moreover, mice arrived at the Institute of Pharmacology and Experimental Therapeutics (iCBR) 1 week previously to the beginning of the protocol for acclimatization and were already marked in the ears and separated into cages. Methamphetamine hydrochloride was purchased to Sigma-Aldrich (St. Louis, Missouri, United States of America) following a special authorization issued by INFARMED Portugal (National Authority for Medication and Health Products I.P.).

3.3. Experimental protocol I

Herein 24 animals were used (Scheme 1). The animals were randomly divided into 4 different groups: METH sedentary (METH/Sed, n=6), METH exercise (METH/Ex, n=6), saline sedentary (Sal/Sed, n=6) and saline exercise (Sal/Ex, n=6). The animals were caged by groups, so that there was no interference between different groups.

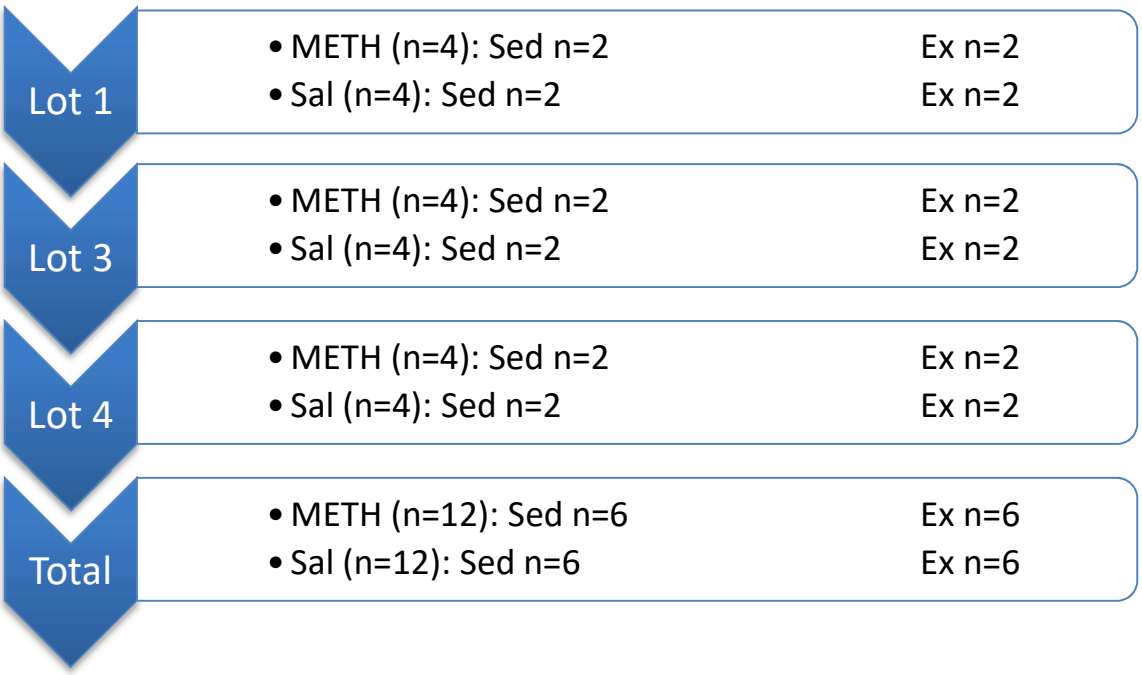
Animals from Lot 1 were housed in pairs in a 2x2x2x2 setting. Animals from Lot 3 were housed in a 1x1x2x2x2 setting. Animals from Lot 4 were housed

in a 2x2x2x1x1 setting. Further modification of the housing settings would happen for bite marks and dominance fights only.

In day 1 animals from the METH groups were administered with a METH solution (30 mg/kg) and the animals from the Sal groups were administered with a saline solution (NaCl 0.9%). Behavioral tests were performed on all the animals 4 weeks (except for Lot 1) and 8 weeks post METH or saline administration. After METH administration, for the next 3 days the animals belonging to the Ex groups were subjected to habituation to a treadmill: mice ran daily at 20cm/s for 20 min and the slope was 8,7% (corresponding to a 5º inclination). The Sed group animals were also placed in the treadmills and left to roam freely during 20 minutes per day.

On the 4th day post-administration the Ex group animals were submitted to an Ergoespirometric test, to individually design the following weeks treadmill protocol.

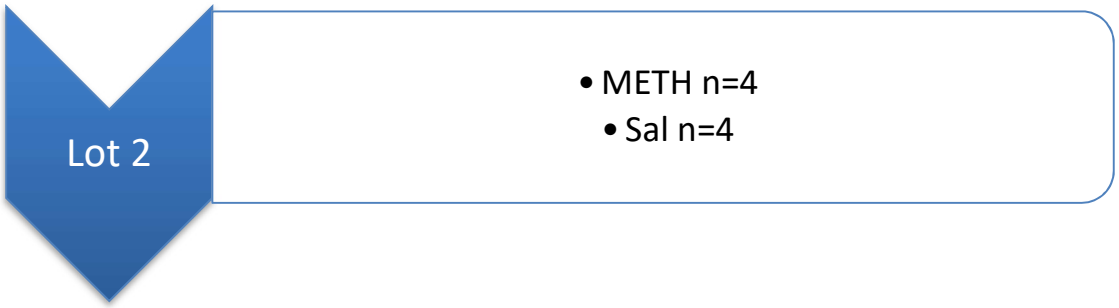
On the last day of the protocol the mice exercised as usual and were sacrificed and biological samples were retrieved.



Scheme 1 Experimental groups for the experimental protocol I

3.4. Experimental protocol II

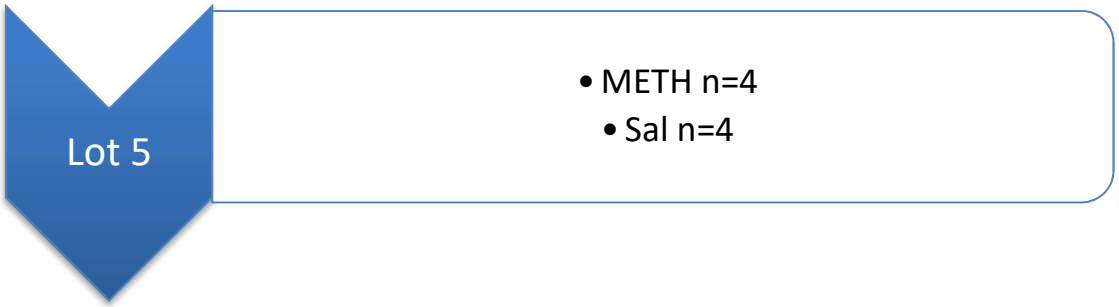
The purpose of this batch of animals (lot 2) was to confirm the induction of a long-lasting depressive-like behavior on METH intoxicated mice, while left sedentary and not daily handled. In this approach the mice were randomly separated into 2 different groups: METH and Saline groups (Scheme 2). After the animals were administered with METH (30/mg/kg) or saline solution (NaCl 0,9%) in day 1 and posteriorly left in the vivarium without daily handling (like Lots 1, 3 and 4), only weekly handling for food/water consumption and weight monitoring. Behavioral tests were performed at weeks 4 and 8. Animals were sacrificed at the end of week 8 and biological samples were retrieved.



Scheme 2 Experimental groups for experimental protocol II

3.5. Experimental protocol III

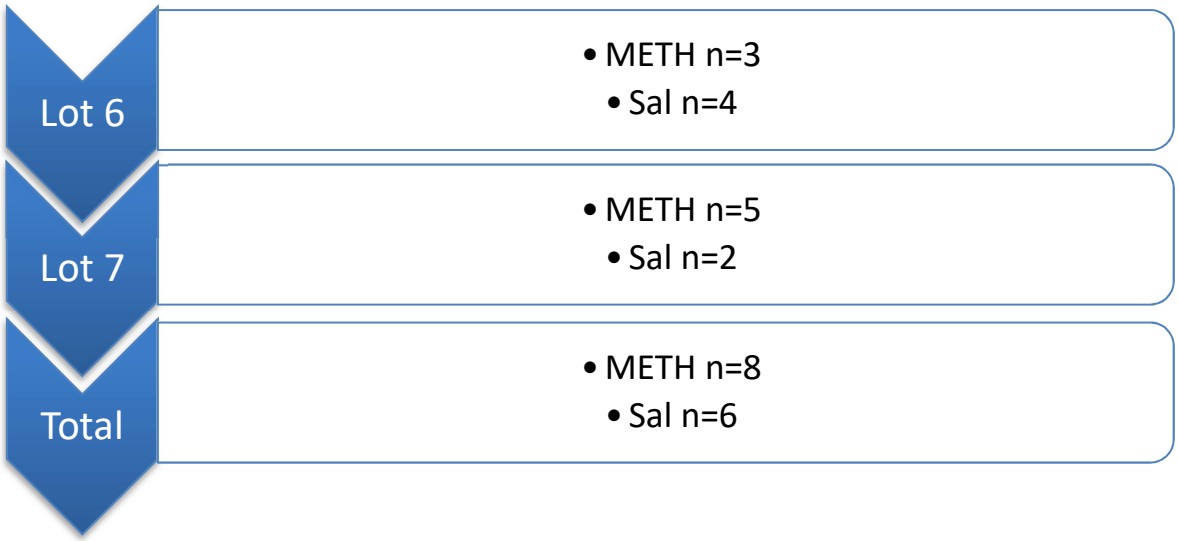
Animals from lot 5 (scheme 3) were used to determine the mid-term emotional outcome of a binge consumption of METH, by administering the same METH dosage as used in previous protocols but with 3 daily administrations (30 mg/kg x 3 days). These animals were subjected to behavioral tests 2 weeks post last METH administration and sacrificed in the day after the last behavioral test, once again biological samples were retrieved. We chose this time-point to be long-enough to allow a time-window for therapeutic intervention and short-enough to see behavioral alterations (2 weeks versus 8 weeks).



Scheme 3 Experimental groups for experimental protocol III

3.6. Experimental protocol IV

Animals belonging to lots 6 and 7 (scheme 4) were used to determine the emotional changes caused by a long-term binge consumption of METH. These animals were subjected to a 2-week administration protocol starting at a 5mg/kg dose in the first week, followed by a 10 mg/kg dose on the second week. Behavioral analysis was performed on the 3rd week of the protocol, which corresponds to the first week after the last administration and on the 5th week of the protocol. This protocol was developed to simulate a near chronic METH consumption. The day after the last behavioral analysis the animals were sacrificed, and biological samples were retrieved. Animals were housed in a 2x2x1x1x1 (lot 6) and 2x2x3 (lot 7) setting.



Scheme 4 Experimental groups for experimental protocol IV

3.7. METH administration protocols

The same METH dosing was used in Experimental design I and II: METH 30mg/kg (3mg/ml) was singly administrated via intraperitoneal (i.p.) injection. The saline animals were administered with a single dose of a NaCl (0.9%; i.p.). The injected saline volumes were similar to METH volumes.

This METH dosing intended to recapitulate the dose used previously by our team. We previously showed that this dose increased immobility time at 3 and 49 days following METH 30 mg/Kg as evaluated by tail suspension test (Silva et al. 2014). We further showed that this METH dosing decreased sucrose-preference and grooming behavior in splash test 1 and 3 days post-METH, respectively (Fonseca et al. 2017). These findings suggested that this METH dosing induced a depressive-like behavior.

All animals after injected were monitored for 4 hours post-injection, to access the gross behavioral changes METH-induced.

Behavior was checked at 15 min, 60 min and 120 min post injection. Animals given METH showed an intense agitation and piloerection at 15 and 60 in post-injection; mice showed an intense grooming in the 15-min observation, tremors were also registered at 60-min measurement as well as a compulsive gnawing in some cases. At the 120-min measurement all signs had disappeared except for the agitation and piloerection which had considerably lessened. Animals from Lot 5 (3-day administration) showed the same behavior as the other Lots. This suggests that there was no tolerance in place.

Except for animal 2 (Lot 1, METH/Ex) that died overnight on the administration day, there were no other casualties due to the administration of such a high METH dosage.

3.8. Treadmill Protocol

The type of physical activity used in the Experimental Protocol 1 (Lots 1,3 and 4) was running in a treadmill. This procedure was performed on a room inside the vivarium at the Institute of Pharmacology and Experimental Therapeutics/FMUC, with controlled conditions (23 ± 1 ºC, low noise and lighting ranging from 5-12 *lux).*

After the habituation to the treadmill (as previously stated; please see page nº 27), the Ex group mice were submitted to an ergoespirometric test, to evaluate their maximum vertical work and to develop individual treadmill sessions according to these ergometric test results.

For the ergoespirometric test the LE 8700 treadmill was used, with a metabolic chamber coupled to a ML206 Gas analyzer (AD Instruments, Bella Vista, Australia) connected to a PL3504 Powerlab (ADInstruments, Bella Vista, Australia) and an ASUS ROG pc with *Labchart* software was used to record and analyze the O_2 consumption and throughout the test. The animal was placed on the treadmill and left to roam freely during a 10 min-accommodation to get a basal reading; then, the treadmill (slope 8,7%) was turned on with an initial velocity of 20 cm/s and we introduced an increase of 5 cm/s every two minutes until the animal could not leave the electrical grid which would deliver electrical shocks to the animal (0,2 mA). When the animal was unable to leave the electrical grid for 5 secs the test was ended. This ergoespirometric test was previously described by Aguiar *et al.* (2018)

The following weeks, exercise was based upon the results of this test. Animals started the week following the ergoespirometric test at 50% of the maximum velocity reached in the test, which is defined by the last velocity the mice could run for 2 minutes, and at the start of all the other weeks there was an increase of 15% of the vertical work performed in the previous week. When the animals failed to comply with the treadmill velocity and tried to rest during the protocol, they were manually stroked to promote movement again, this approach was used instead of applying electric shocks.

The vertical work (VW; Nm) was calculated according to the following formula: $VW = (w * g) * v * t * s$; *w* is the weight of the animal in kilograms, *g* is the standard gravity, *v* is the velocity in m/s, *t* is the time (s) and *s* is the slope (sine

of the treadmill angle [0.087]) (Katch, McArdle and Katch, 2013). In the ergoespirometric test, the vertical work is the sum of the work performed by mice in all tested velocities.

The treadmill protocol was always done in the morning period, from 9am to 2pm, except on the days of the behavioral tests in which the exercise was done from 3pm to 6pm.

To standardize the stress and handling conditions and frequency, the sedentary mice were always put on the treadmills (while they were off) and were allowed to freely roam during the same time as the exercised animals.

3.9. Behavioural assessment

To access the possible behavioral changes produced by the METH and/or the physical exercise all the mice were submitted to Open Field test (OFT), Tail-Suspension Test (TST), Splash Test (ST) and Forced-Swim Test (FST). The tests were performed on days 17 and 18 (Lot 5), 24 and 25 (Lots 3, 4 and 2) and on days 52 and 53 (Lots 1,3 and 4 and Lot 2), and on days 16 and 18 and 30 and 32 (Lots 6 and 7).

The tests were performed on a specific room at the Institute of Pharmacology and Experimental Therapeutics, at controlled lighting settings. The animals were placed in the room 30min previously to the beginning of the tests and left unperturbed and at low light.

Due to camera and memory card issues, some videos of the behavioral tests could not be used, therefore changing the expected number of animals in certain groups.

3.10. Open Field Test

The open field test was performed under 8-9 *lux* of white light. This test can measure mice locomotor activity and their anxiety-like behavior (Tatem *et al.*, 2014; Kraeuter, Guest and Sarnyai, 2019; .Heredia *et al.*, 2014; Kuniishi *et al.*, 2017).

For this test 2 different apparatus were used: square and circular open fields. The square open-field was always the first one used (week 4) and the circular one was used at the second test (week 8) to prevent carryover or learning effects. The square open field has the following dimensions: 45x45x45cm and the circular one has 41cm of diameter (at the bottom) and has a depth of 40cm. Two distinct zones were defined by the *Anymaze* software: the periphery and the center, which was defined as the central 30% of the open field area available for the animals (either square or circular depending on the apparatus).

The test has a 10-minute duration and 7 parameters were evaluated, 4 for locomotor activity and 3 for anxiety-like behavior(Seibenhener and Wooten, 2015). For locomotor activity the total distance travelled, the total mean speed, total time immobile and the number of immobile episodes were chosen, and time spent in center, distance in center and distance percentage in center were selected(Heredia *et al.*, 2014; Seibenhener and Wooten, 2015) for assessing the anxiety-like behavior.

3.11. Tail Suspension Test

The tail suspension test is used to assess the anti-depressant effect of compounds on mice and to evaluate any procedure that can lead to the development of depressive-like behavior (Rial *et al.*, 2014). This test relies on the fact that mice when subjected to a stressful situation will (or not) try to escape the situation. However, since they cannot escape, they adopt a still posture. The mice were individually hanged from their tails in a height of 50 cm and their behavior recorded for 6 min. Data retrieved from this test was the following: 1) latency it took

for each mouse to give up on trying to escape and to adopt an immobile posture (Latency Time) and the total time the mouse stayed hung passively during the 6 min (Immobility Time).The test was performed under 17 *lux* (white light) and the mice were hung separately and left alone in the room for the duration of the test. The recorded test was then analyzed by the team.

3.12. Splash Test

The Splash Test consists of evaluating the self-care and motivational behavior in the mice, as it is known to be decreased in people suffering from MD, by squirting the mice's back with a sucrose solution (10%v/v) and recording the grooming (act of the mouse cleaning its own coat) for the next 5 minutes (Isingrini *et al.*, 2010).

For this test the mice were recorded in their own cages, alone, with 8 *lux* of white light. The recordings were then analyzed by the team.

3.13. Forced-Swim Test

The Forced-Swim Test is widely used to access depressive-like behavior in mice, as similarly to the Tail Suspension Test. In this test, mice are put in a threatening situation: in fact, mice don't do well in water, although they can swim.

Therefore, this test consists in putting the mice in a cylindric tube (with approximately 11 ± 1cm of diameter) filled with water, deep enough so the mice can't touch the bottom of the tube. This is made so they don't feel any form of comfort, also the water temperature was controlled and adapted to not cause any relaxation (24±1ºC). The test has a 6-minute duration which was recorded under a lighting of 17 *lux* (red light) and the video was analyzed by the team. After the test

the animals were dried before they returned to their cages. The data retrieved from this test is the time at which the animal gives up trying to escape (Latency Time) and starts floating only and the total immobility time (time spent only floating with no clear intent of escaping) (Rial *et al.*, 2014).

3.14. Animal Sacrifice

The animals belonging to the Ex groups were euthanized 10 minutes after finishing their exercise protocol. For the euthanasia, first the animals were subjected to isoflurane (IsoFlo, Zoetis; inhalation) and then to a ketamine/xylazine solution (100mg/kg/10 mg/kg; i.p). Blood, heart, thymus, spleen, adrenal glands and brain regions (striatum and frontal cortex) were also retrieved for further study.

3.15. Data analysis

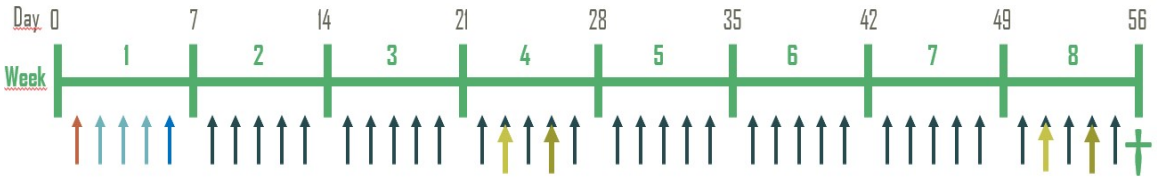
Results were expressed as mean ± SD. Groups were compared using Two-Way ANOVA test corrected for multiple comparison tests using the Bonferroni's test. An unpaired, two-tailed student´s t-test was used when appropriate. Differences were considered to be significant at $p<0.05$. Statistical analyzes were performed using the software GraphPad Prism 7.0.

4. Results

4.1. Experimental Protocol: Single-high METH dose followed by a 8-week treadmill protocol

The animal's weight and food consumption were monitored weekly during the experiment. In fact, the animals and the food were weighted to access eventual changes related to the protocols, every Monday.

Animal 32 (Sal/Ex group) died in a treadmill related accident on 3 days prior to the end of the protocol, so it could not be included in some of the behavioral analysis at week 8, therefore changing the number of animals expected on that group.



Scheme 5 Experimental protocol I: Orange arrow: Meth administration (single i.p. 30mg/kg) day; Light Blue arrows: Habituation to the treadmill; Blue arrow: Ergoespirometric test; Dark green arrows: treadmill protocol; Light green arrows: Behavioral tests; Green cross: Euthanasia;

4.2. Body weight and food consumption

The body weight of all the animals changed during the course of the experiment ($p<0.0001$) (Figure 4A). Additionally, neither PE nor METH significantly changed the percentages of body mass gain throughout the experimental protocol. However, one should stress that METH shows a tendency to decrease the weight gain regardless of exercise (Figure 4B).

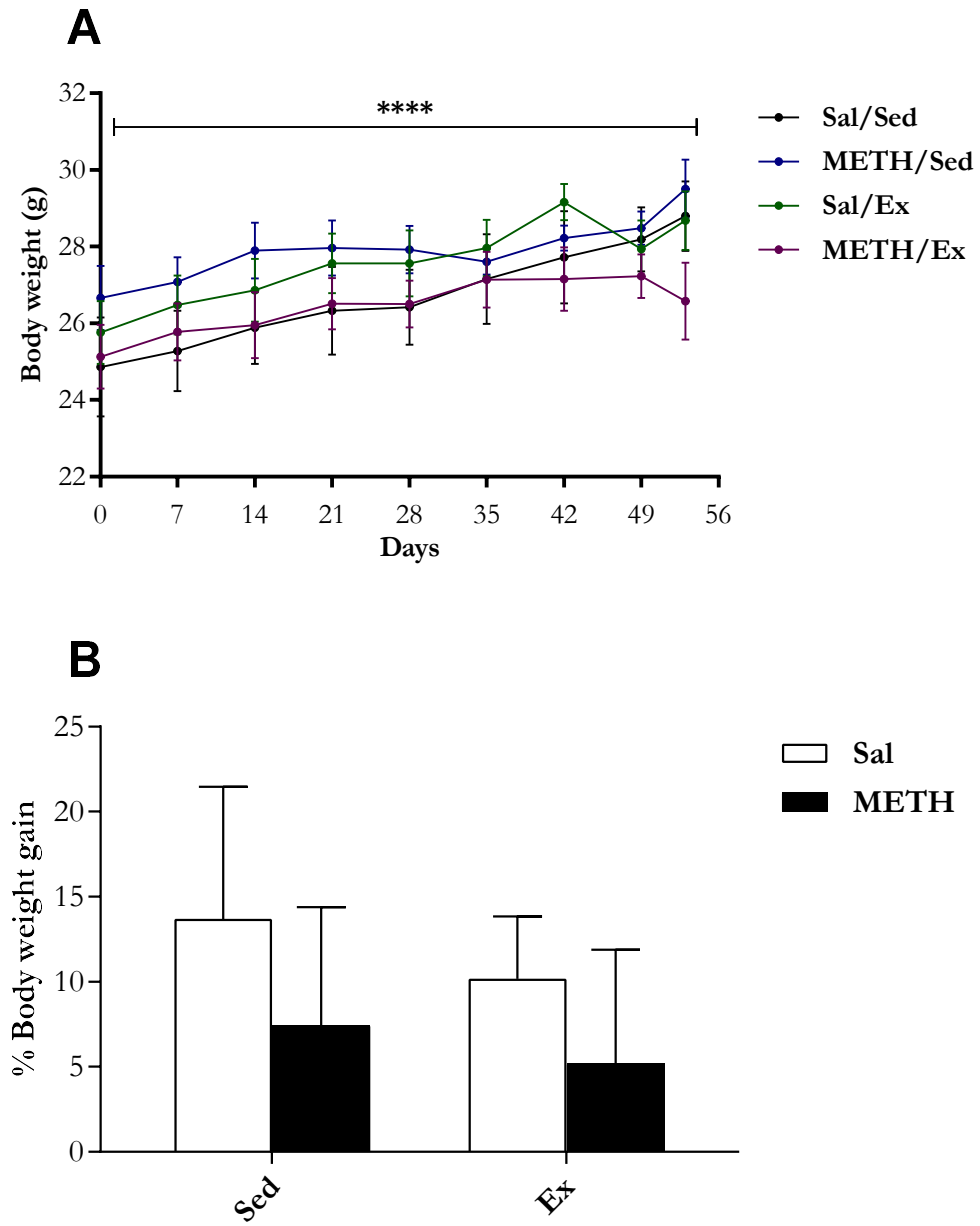


Figure 4. **A**: Evolution of body weight of animals from the 4 groups over the total duration of the experiment (54 days); **B**: Percentage of body weight gained on during the experiment. Sal/Sed n=6; Sal/Ex n=5; METH/Sed n=5; METH/Ex n=6. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; *p<0.0001.

Food consumption is presented as the average food consumption per day and per animal (Figure 5). No difference was observed between groups. No effect of METH administration was recorded. Additionally, physical active mice seem to ingest the same amount of food when compared to sedentary mice.

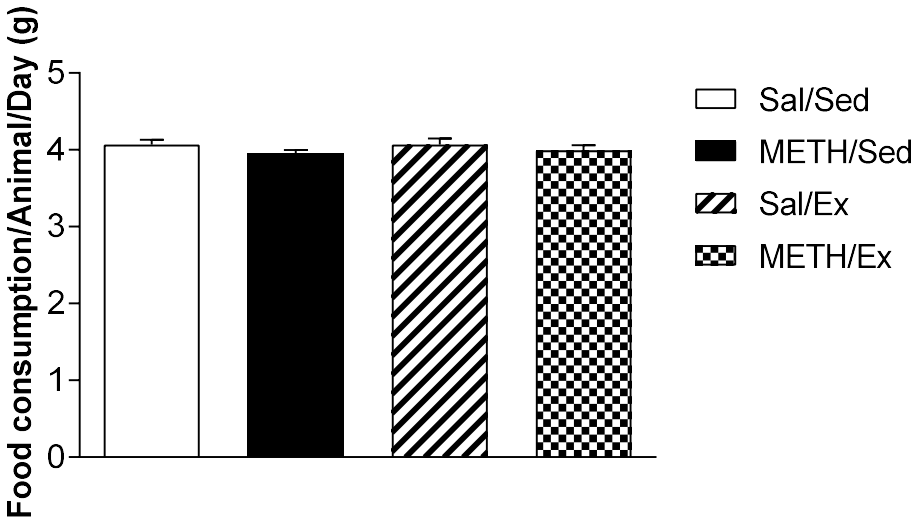


Figure 5. Average food consumption per day per animal. Sal/Sed n=6; Sal/Ex n=5; METH/Sed n=5; METH/Ex n=6. Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

4.3. Ergoespirometric test

The Ergoespirometric test was performed 4 days after the METH administration, and METH-mice showed no effects on the analyzed parameters. Particularly, there were no significant differences in the METH *vs* Sal groups tested in the total vertical work and the exhaustion time (Figure 6). This is in contrast with data presented by Morozova *et al.* (2016) showing that amphetamine administration leads to an improved physical performance in athletes right after drug administration. It seems that the 4 day gap between the METH administration and the Ergoespirometric test nullified METH ergogenic effect.

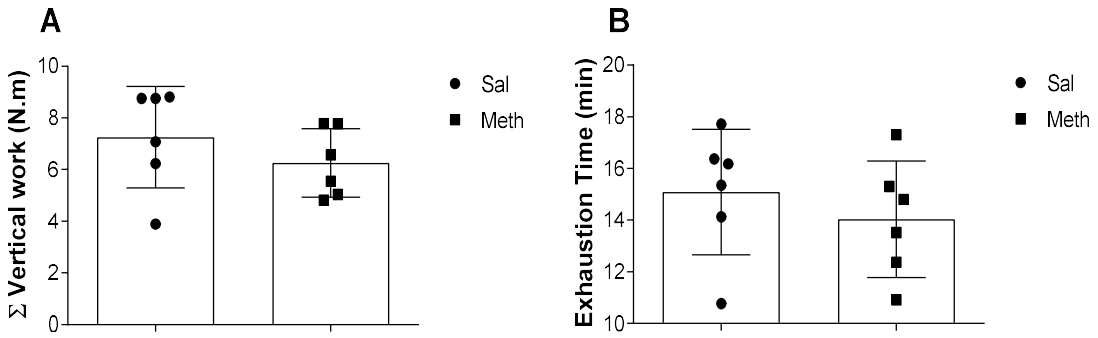


Figure 6. **A**: The total vertical work performed by METH and Sal groups in the ergoespirometric test; **B**: Exhaustion time of METH and Sal groups in the ergoespirometric test). Sal n=6; Meth n=6. Data are presented as mean ± SD and were analyzed by an unpaired two-tailed student´s t-test; p>0.05.

The VO_{2max} uptakes were also not affected by the METH administration as Figure 7 illustrates.

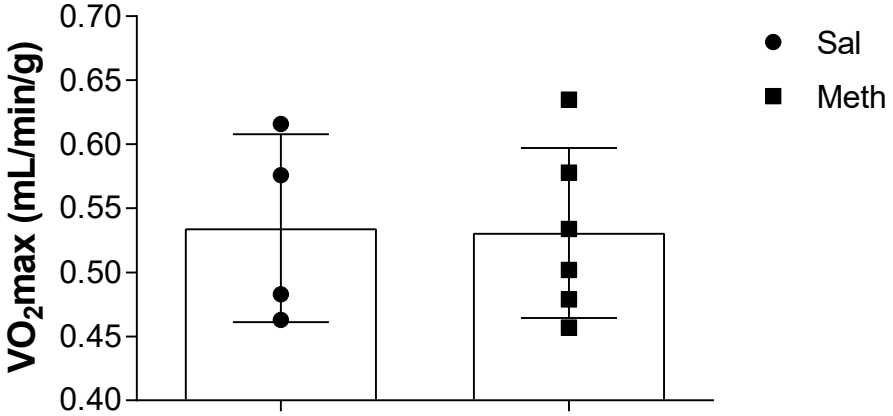


Figure 7. Oxygen consumption during the Ergoespirometric test. Sal n=4; METH n=6. Data are presented as mean ± SD and were analyzed by an unpaired two-tailed student´s t-test; p>0.05.

The vertical work realized in the treadmill sessions was significantly increased from the beginning to the end, meaning that it was significantly different over the time course of the experiment. However, there was no difference between the METH and Sal groups in terms of vertical work per session (Figure 8).

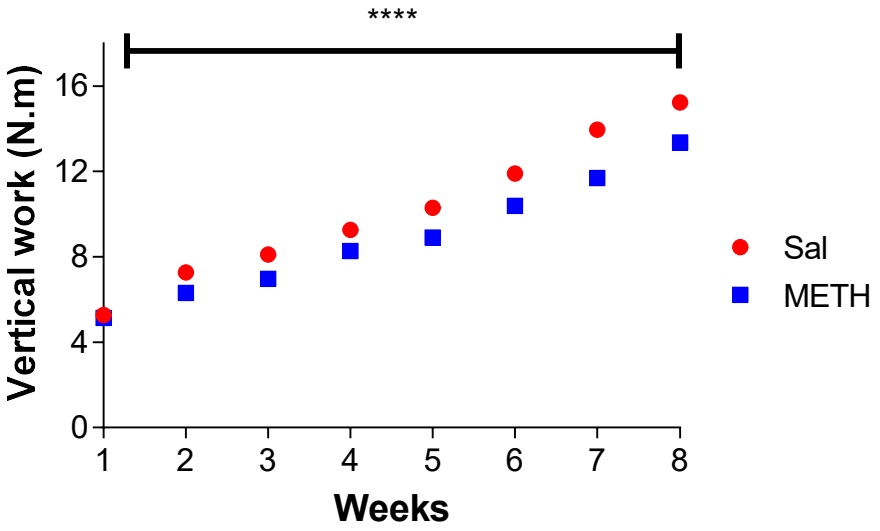


Figure 8. Evolution of the vertical work per week in each treadmill session. Sal n=6; METH n=6. Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; *p<0,0001

4.4. Emotional fingerprint of METH-intoxicated mice subjected to 4 weeks of physical exercise

Open Field Test

Neither physical exercise nor METH significantly changed the general activity level parameters assessed in an Open Field square at week 4, during a 10 min. evaluation period. In fact, all groups showed similar total distance travelled (Figure 9A), total mean speed (Figure 9B), total time immobile (Figure 10A) and number of immobile episodes (Figure 10B).

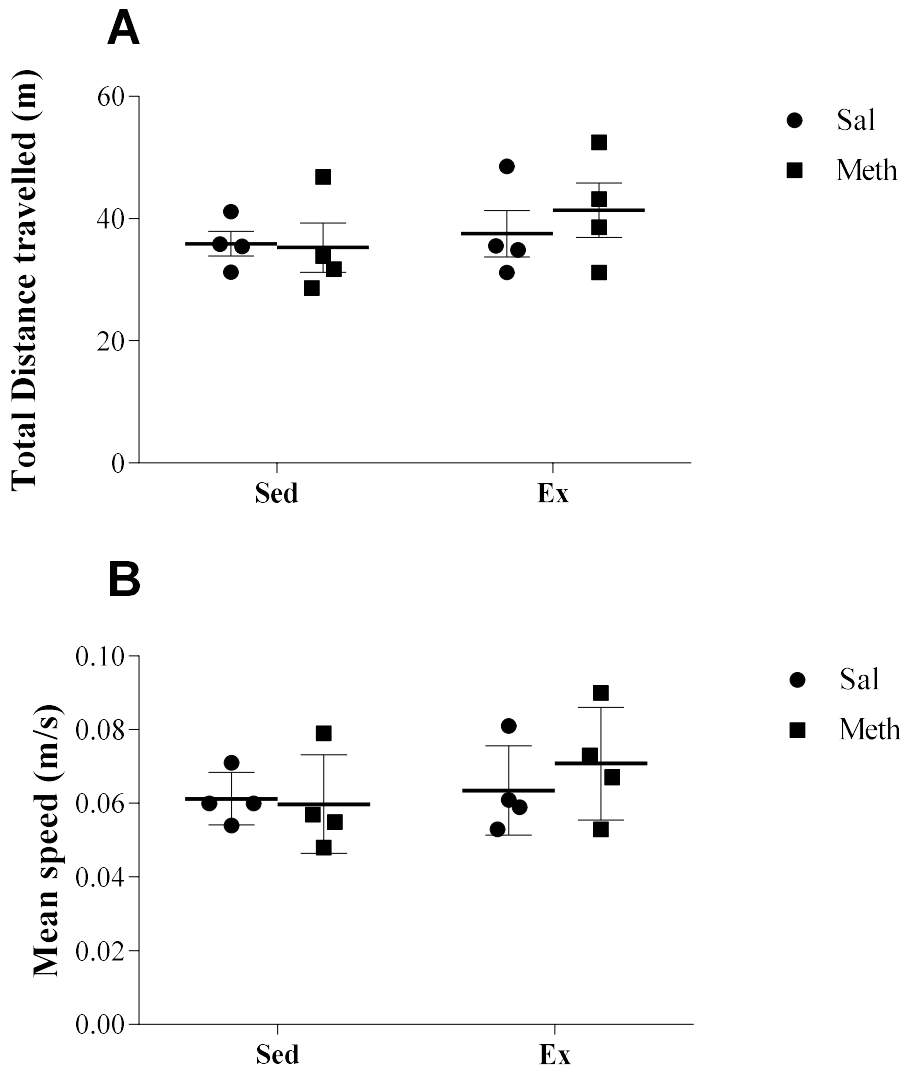


Figure 9. Behavioral analysis on week 4 in a Square Open Field. **A**: Total Distance travelled (m); **B**: Mean speed (m/s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Since the center part of the OF arena is aversive, the time and distance travelled in this area are used to assess anxiety-like behavior in rodents (Struntz and Siegel, 2018). Immobility can also be used as a measure of anxiety as contrasting to the exploratory activity inherent to the mice; therefore, time and number of episodes of immobility can be also measured to this purpose(Seibenhener and Wooten, 2015). Herein, we couldn't find any differences in the distance in the center (Figure 11A), in percentage of distance in the center

(Figure 11B) and in the time spent in the center (Figure 11C) between four groups.

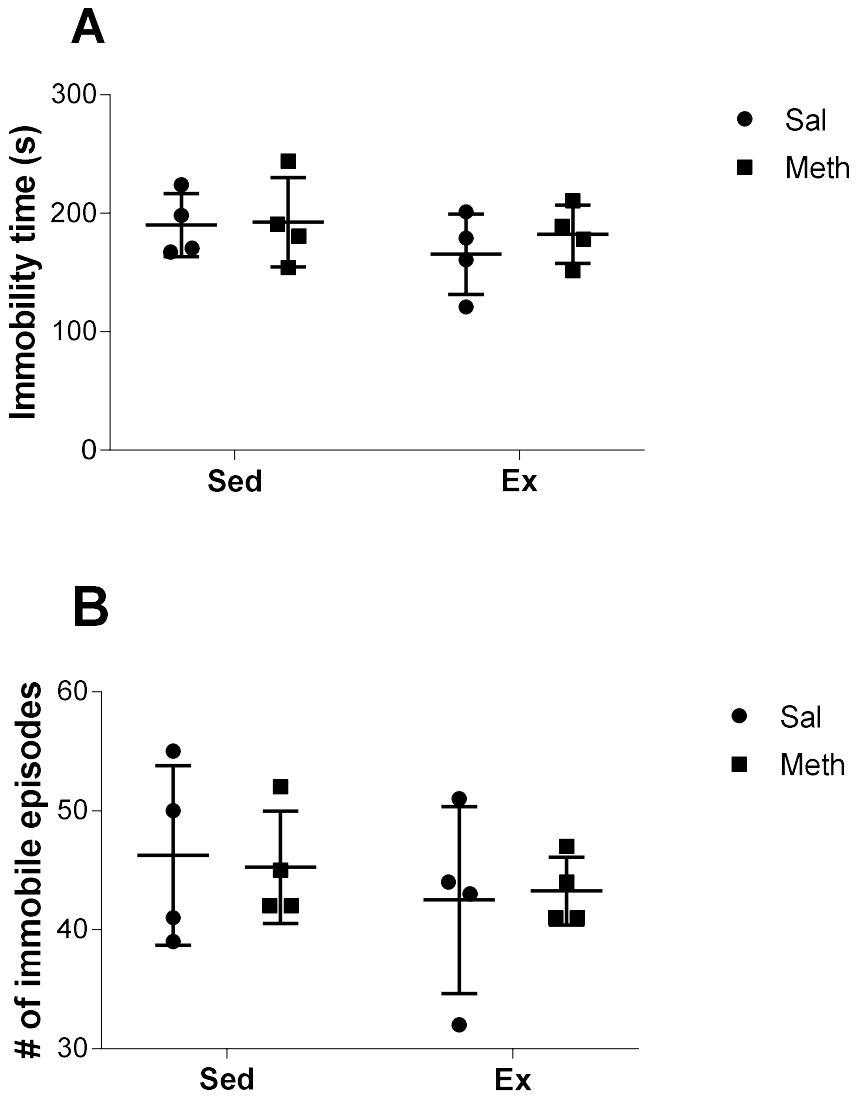


Figure 10. Behavioral analysis on week 4 in a Square Open Field. **A**: Total immobility time (s); **B**: Number of immobile episodes. Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

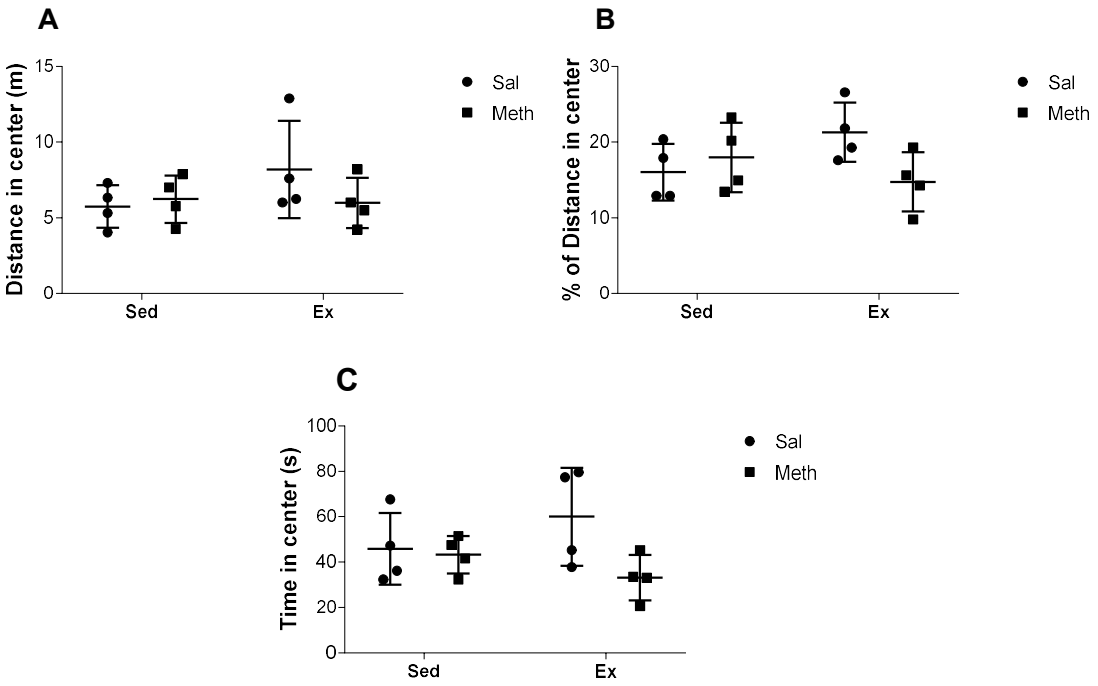


Figure 11 Behavioral analysis on week 4 in a Square Open Field. A: Distance travelled in center (m); B: Percentage of total distance travelled in the center (%); C: Time spent in center (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Tail Suspension Test

In the Tail suspension test the latency to immobility (Latency Time) and the total time of immobility (Immobility Time) during the 6 minutes of the test were measured.

Nor the METH administration, nor the physical exercise protocol exerted visible effects on the behavior of the animals at week 4 (Figure 12). The METH-mice showed no signs of depressive-like behavior, contrary to what was demonstrated by Silva *et al.*, (2014).

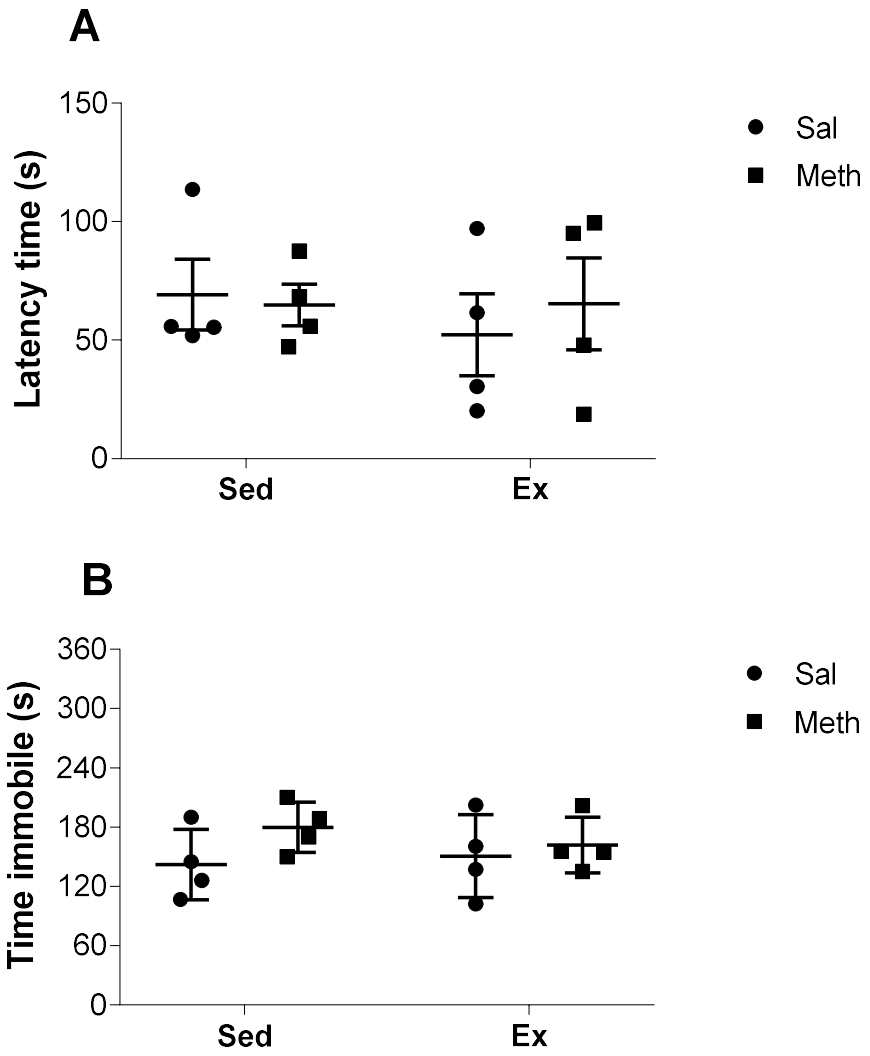


Figure 12. Behavioral analysis on week 4 using Tail Suspension test. **A**: Latency to immobility (s); **B**: Total time spent immobile (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Splash Test

In the splash test, the data retrieved from the mice showed no changes in the grooming behavior as shown in Figure 13. In fact, neither METH nor PE significantly changed latency grooming and total grooming time (Figure 13A, 13B, respectively).

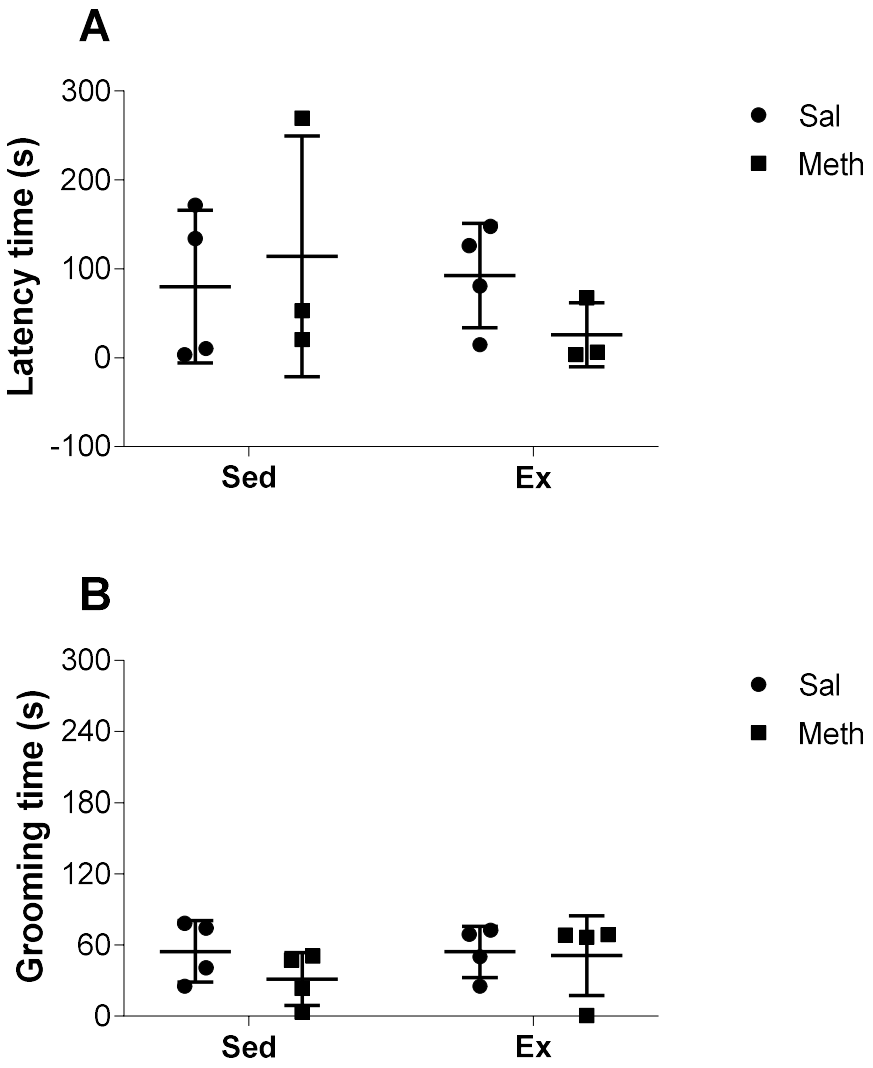


Figure 13. Behavioral analysis on week 4 using Splash test. A: Latency time to dorsal grooming (s); B: Total grooming time (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Forced-Swim test

In the forced-swim test the animals also showed no differences between groups, given that neither the METH- nor the PE-groups showed any effects over the control groups in week 4, as the latency for immobility (Figure 14A) and the total immobility time (Figure 14B) were not significantly different between groups.

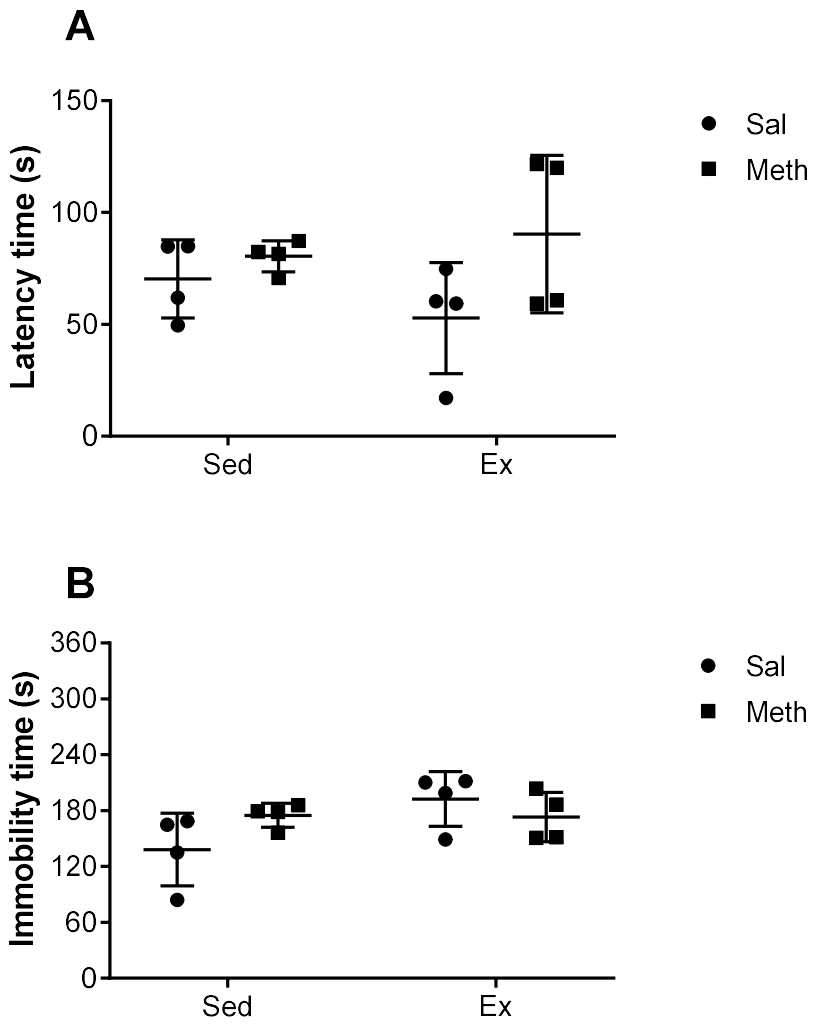


Figure 14. Behavioral analysis on week 4 using Forced-swim test. **A**: Latency time to immobility (s); **B**: Total immobility time (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

4.5. Emotional fingerprint of METH-intoxicated mice subjected to 8 weeks of physical exercise

Open field test

The METH and the exercise groups continued to show no statistical differences in the total distance travelled (Figure 15A) and in the total mean speed (Figure 15B). METH and the physical exercise also did not evoke any statistically significant effect on the immobility parameters of mice in the 8th week of the OFT (Figure 16A, B).

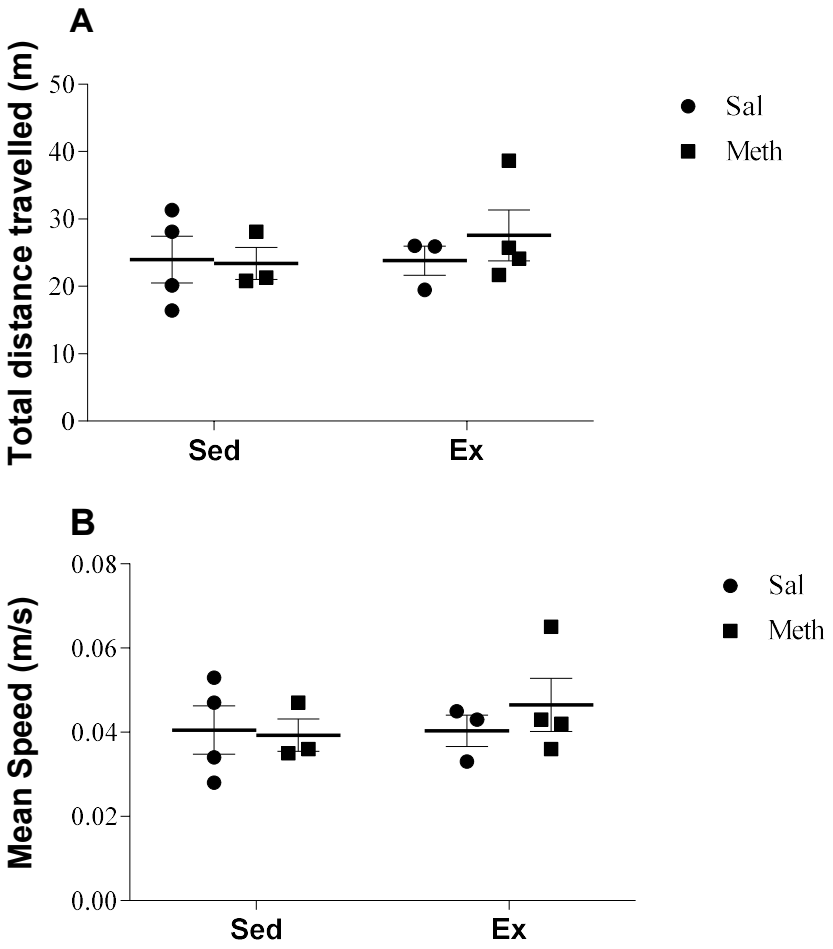


Figure 15 Behavioral analysis on week 8 in a Circular Open Field. **A**: Total Distance travelled (m); **B**: Total Mean speed (m/s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=3-4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; p>0.05.

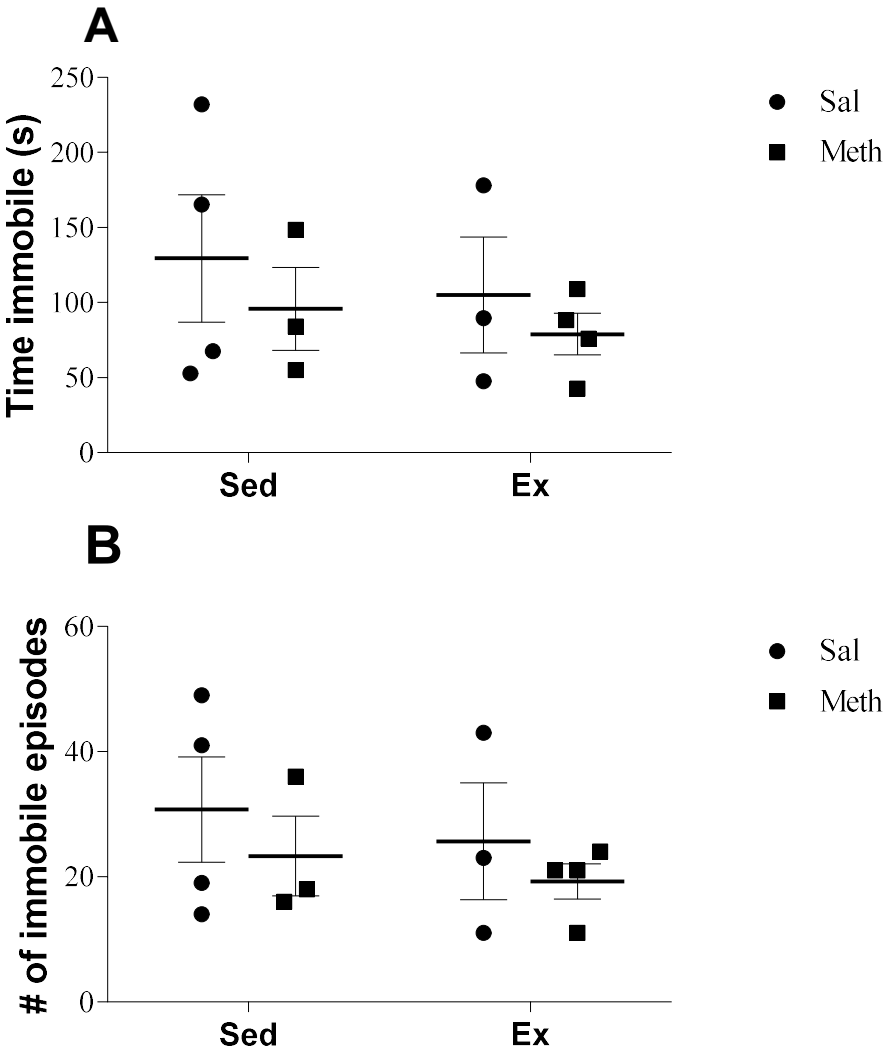


Figure 16 Behavioral analysis on week 8 in a Circular Open Field. **A**: Time immobile (s); **B**: Number of immobile episodes. Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=3-4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Regarding the Center parameters (distance in center; %distance in center; time in center), there were no differences between groups in week 8 (Figure 17A-C), similarly to behavior displayed in week 4.

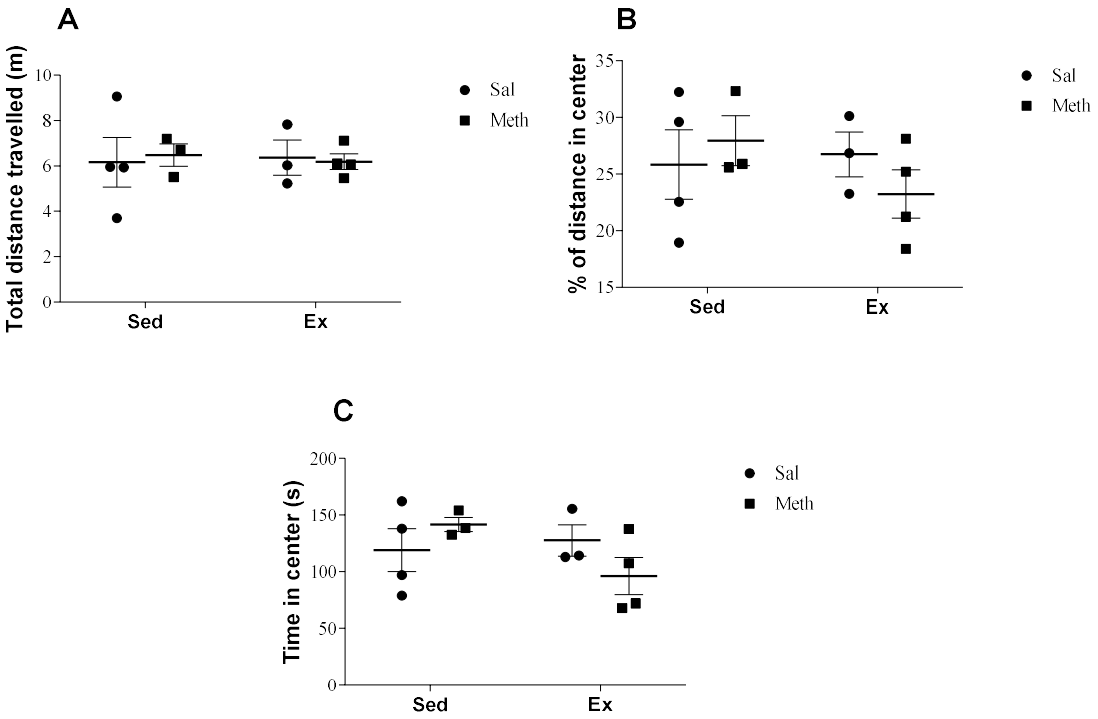


Figure 17. Behavioral analysis on week 8 in a Circular Open Field. **A**: Total Distance Travelled in center (m); **B**: Percentage of distance travelled in center (%); **C**: Time spent in center s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=3-4). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Tail suspension test

Similarly to the behavioral profile evaluated at week 4, no significant differences were found between groups in week 8 regarding latency to immobility and immobility time in TST, as shown in Figure 18 (A, B).

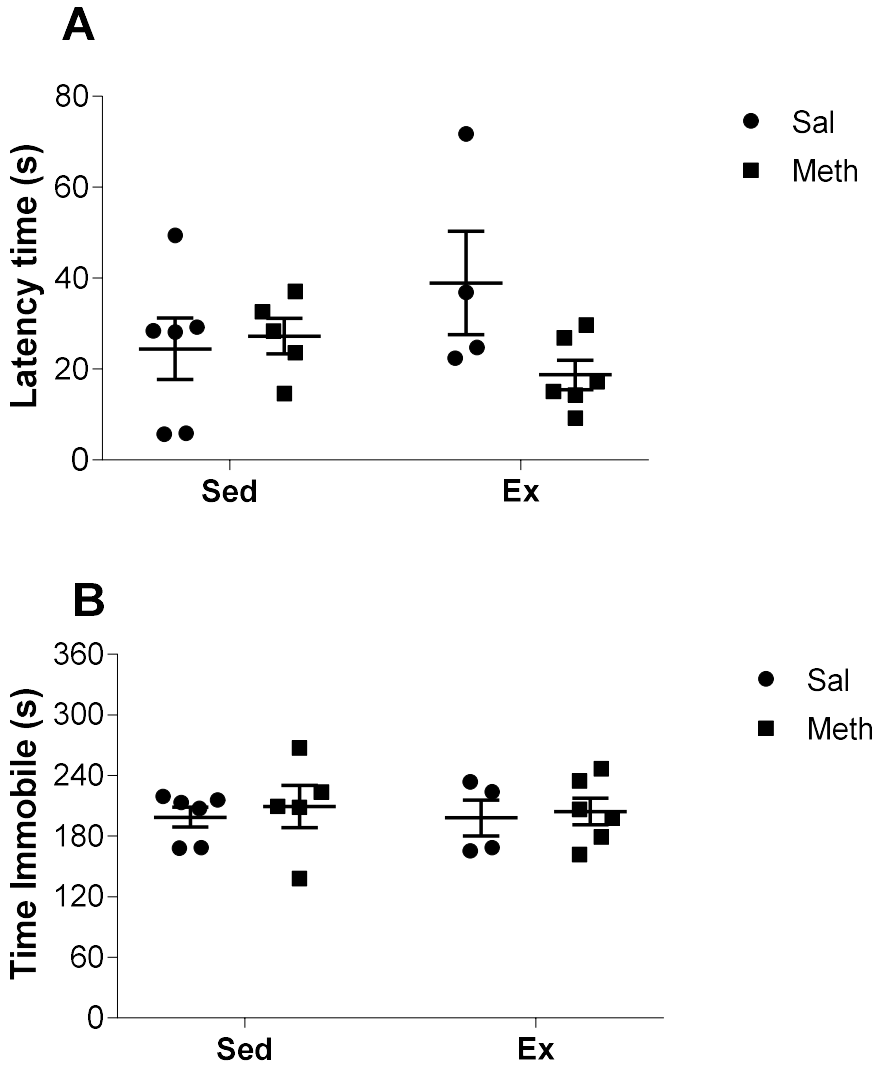


Figure 18. Behavioral analysis on week 8 using Tail Suspension test. A: Latency to immobility (s); B: Total time spent immobile (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4-6). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Splash test

The splash test analysis showed that neither METH nor PE exerted any significant effect on latency time to dorsal grooming and total grooming time at the week 8 of the protocol (Figure 19A, B).

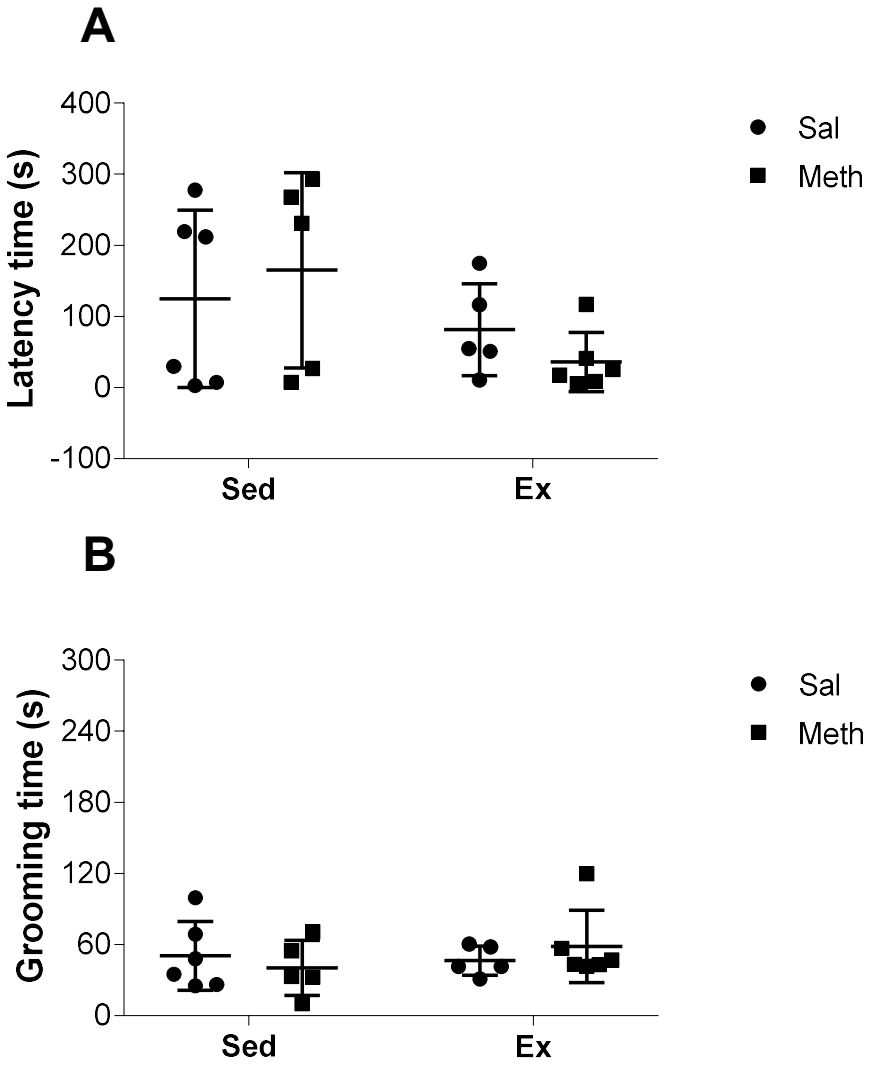


Figure 19. Behavioral analysis on week 8 using Splash test. A: Latency time to dorsal grooming (s); B: Total grooming time (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=5-6). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

Forced Swim test

In the forced swim test the animals also showed no differences between groups, given that neither the METH- nor the PE-groups showed any statistically significant differences when compared with the respective controls: the latency for

immobility (Figure 20A) and the total immobility time (Figure 20B) remain non-significantly different from controls.

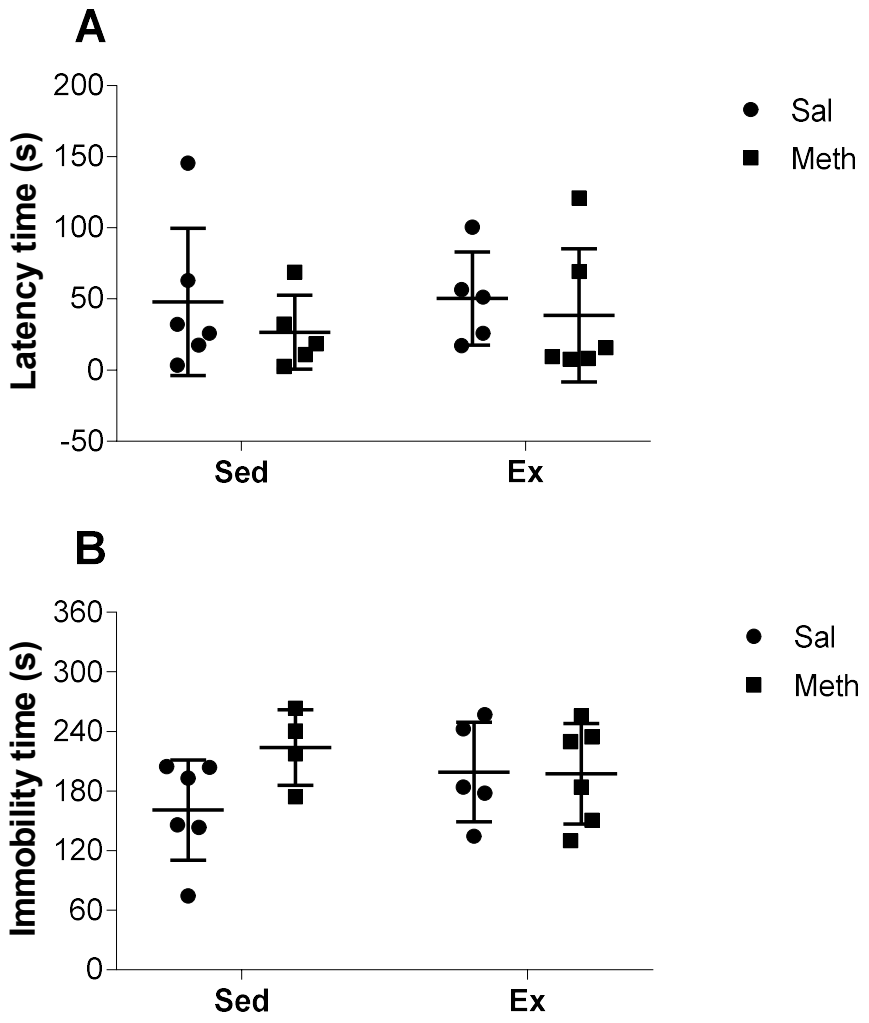
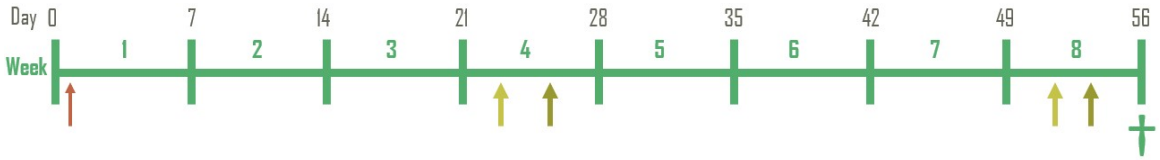


Figure 20. Behavioral analysis on week 8 using Forced Swim test. **A**: Latency time to immobility (s); **B**: Total immobility time (s). Sal/Ex, Sal/Sed, METH/Ex, METH/Sed (n=4-6). Data are presented as mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; $p>0.05$.

4.6. Experimental Protocol II

The animals belonging to the Experimental Protocol II were subjected to a single METH administration and then left in the vivarium with minimal handling, to check if the daily handling of the sedentary animals in the exercise protocol and exposure to the treadmill apparatus could be masking the depressive symptoms of the METH administration. Only data from week 4 behavioral analysis is presented, due to the data of the week 8 being still under analysis.



Scheme 6. Experimental protocol II: Orange arrow: Meth administration (single i.p. 30mg/kg) day; Light green arrows: Behavioral tests; Green cross: Euthanasia.

Body Weight

The weight of the animals changed during the course of the protocol, but METH administration produced no difference in weight between groups.

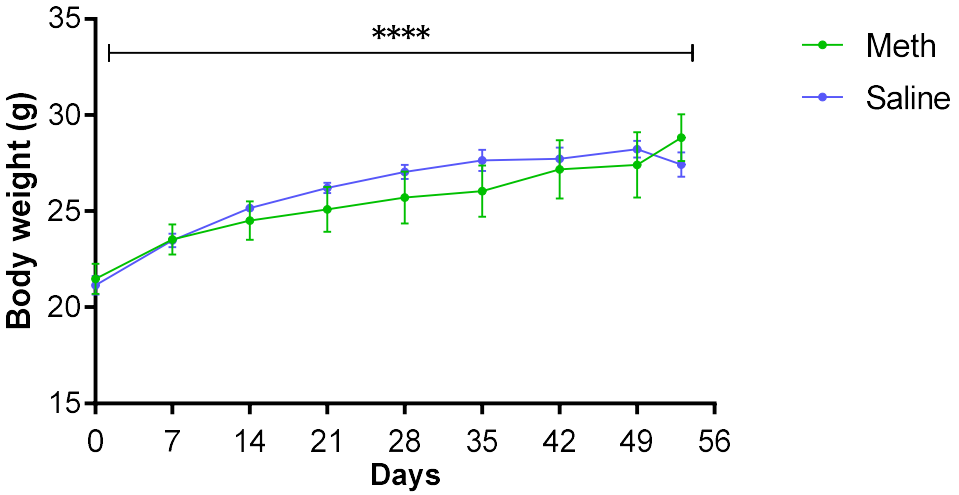


Figure 21 Evolution of body weight of animals from the groups over the total duration of the experiment (54 days); Meth n=4; Saline n=4. Data are presented as mean

± SD. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; *p<0.0001

4.7. Emotional fingerprint of METH-intoxicated mice at 4 weeks after a single high dose of METH

Open field test

In the open field test, the same parameters used in Experimental protocol I analysis were analyzed total distance travelled, total mean speed, time in center, distance in center, percentage of distance in center, time immobile and immobile episodes. Regarding the locomotor parameters, it is shown that METH (30 mg/kg) does not significantly change total distance travelled, total mean speed (Figure 22A, B) and in the time spent immobile and the number of immobile episodes (Figure 23 A, B).

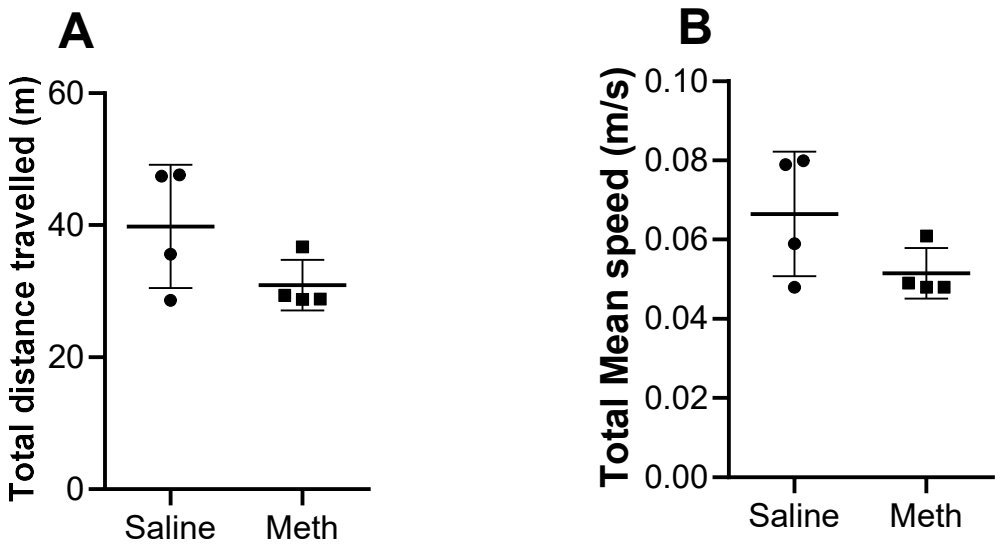


Figure 22. Behavioral analysis in week 4 in a Square Open Field. **A**: Total Distance travelled (m); **B**: Total Mean speed (m/s). Sal, METH (n=4). Data are presented in mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

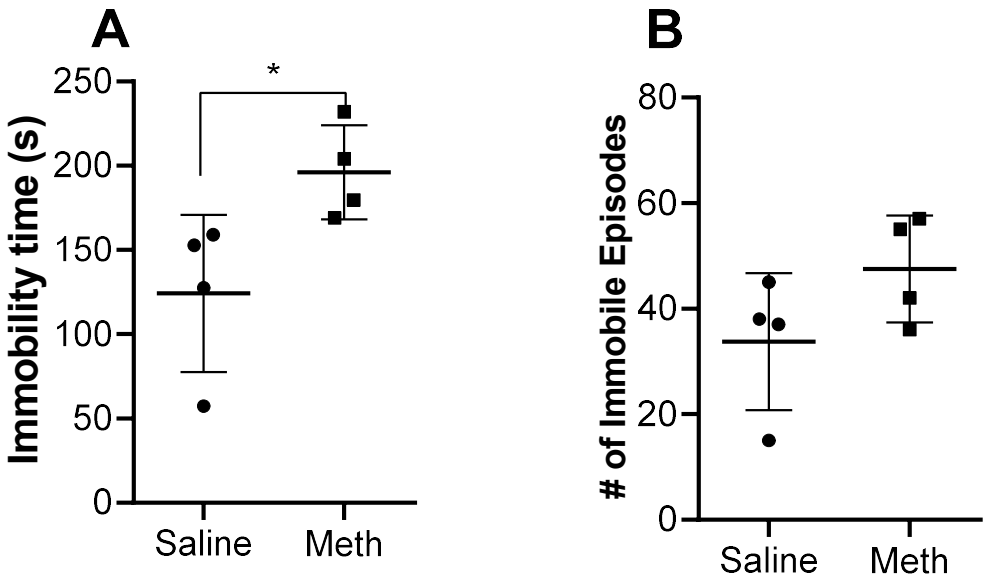


Figure 23 Behavioral analysis in week 4 in a Square Open Field. **A**: Total immobility time (s); **B**: Number of immobile episodes. Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by was analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

The anxiety-like behavior parameters (distance in center; % of distance in center and time in center - Figure 24A-C) remained non-significantly different between the METH-intoxicated mice and the control group.

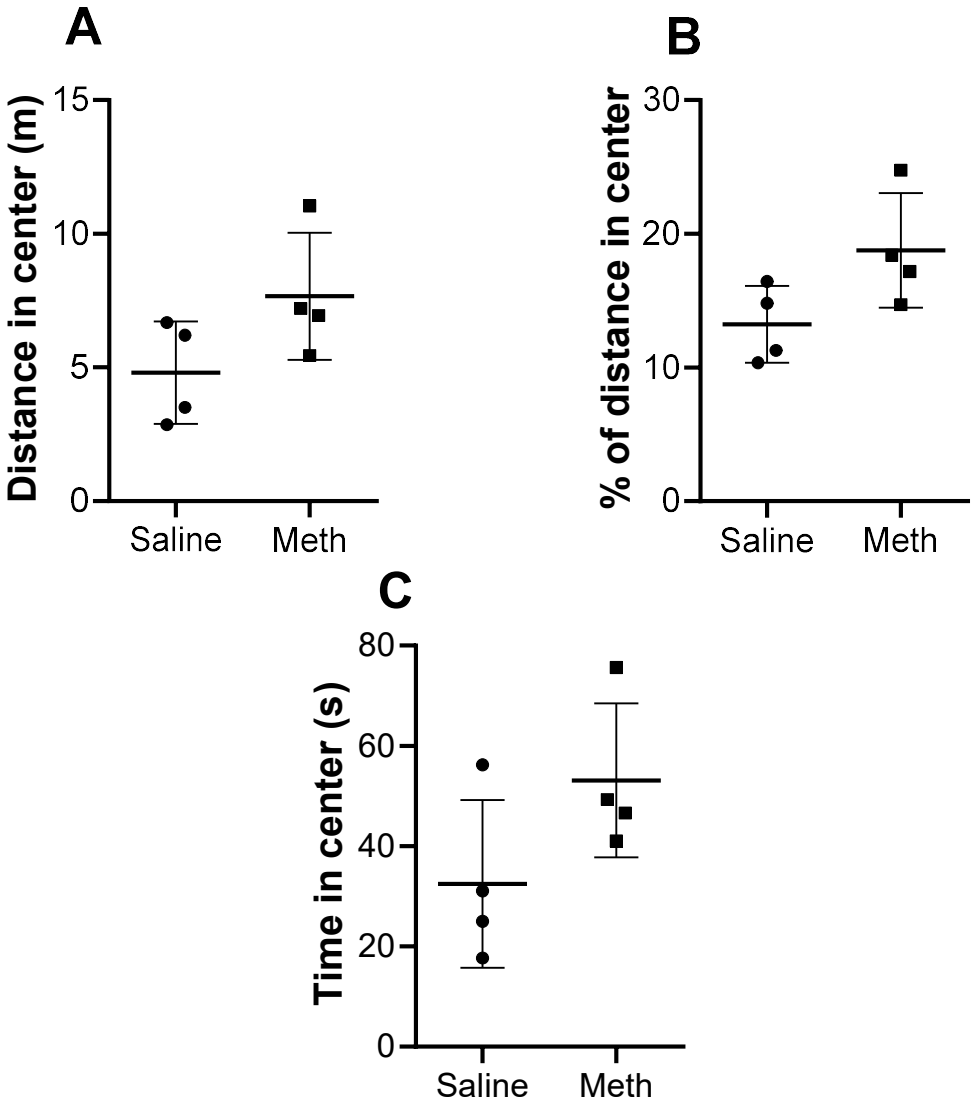


Figure 24 Behavioral analysis in week 4 in a Square Open Field. A: Distance travelled in center (m); B: Percentage of total distance travelled in the center (%); C: Time spent in center (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Tail Suspension test

In the Tail Suspension test, the latency time to immobility (Latency time) and the total time mice spent immobile (Immobility time) were analyzed.

The intoxicated mice did not show statistically significant differences when compared to saline controls (Figure 25A, B).

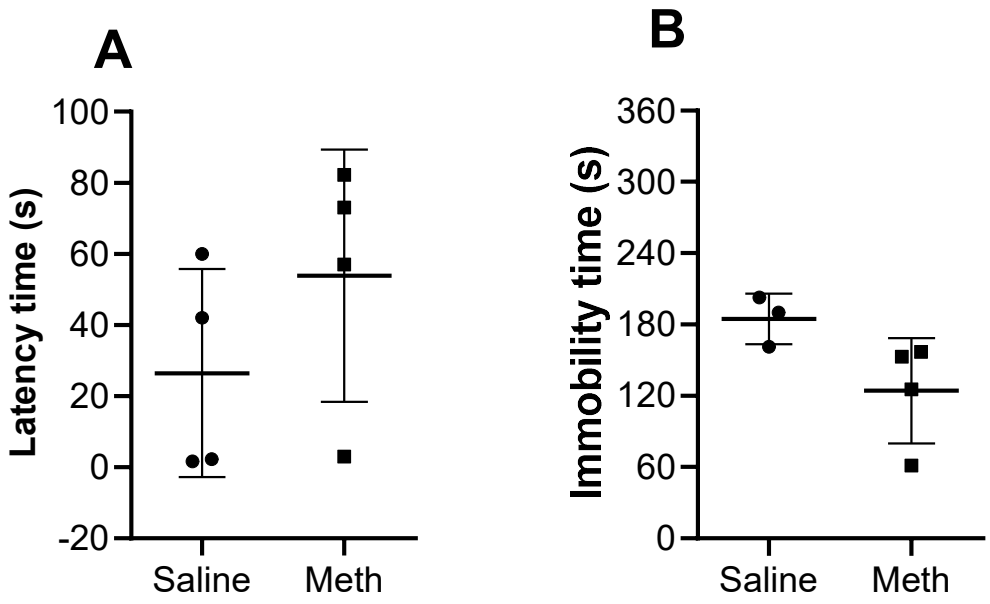


Figure 25. Behavioral analysis in week 4 using Tail Suspension test. **A**: Latency to immobility (s); **B**: Total time spent immobile (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

Splash test

In the Splash test, there were also no statistically significant differences between the latency to dorsal grooming and the total grooming time between METH and Saline mice (Figure 26).

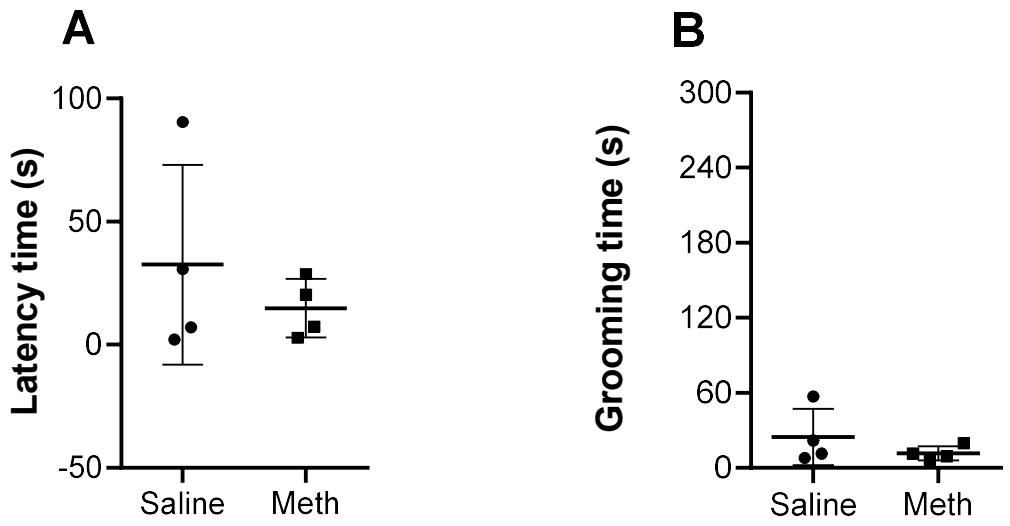


Figure 26. Behavioral analysis in week 4 using Splash test. **A**: Latency time to dorsal grooming (s); **B**: Total grooming time (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Forced Swim test

The latency to immobility (Figure 27A) and the total immobility time (Figure 27B) were measured during the forced swim test. No differences were found between groups, as shown in Figure 26A, B.

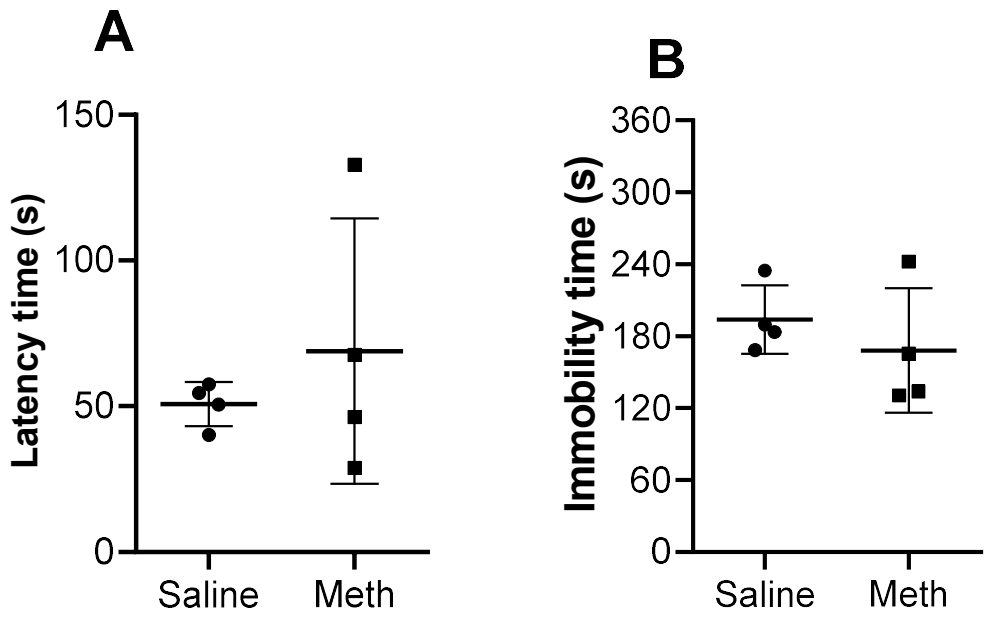
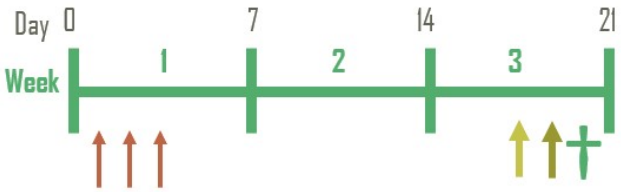


Figure 27. Behavioral analysis in week 4 using Forced Swim test. **A**: Latency time to immobility (s); **B**: Total immobility time (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

4.8. Experimental protocol III

As both Experimental protocols I and II animals failed to develop both anxiety and depressive-like behaviors, in Experimental protocol III 3 daily doses of METH (30 mg/kg, i.p.) were administered and the animals were tests 15 days after the last drug injection, to test if this binging dosage could lead to a depressive- or an anxiety-like behavior.



Scheme 7. Experimental protocol III: Orange arrows: Meth administration (daily i.p. 30mg/kg) day; Light green arrows: Behavioral tests; Green cross: Euthanasia.

Body Weight

Animals from this protocol didn't have their body weight affected by the METH administration, only had a natural body weight gain from the passage of time, as shown in Figure 28.

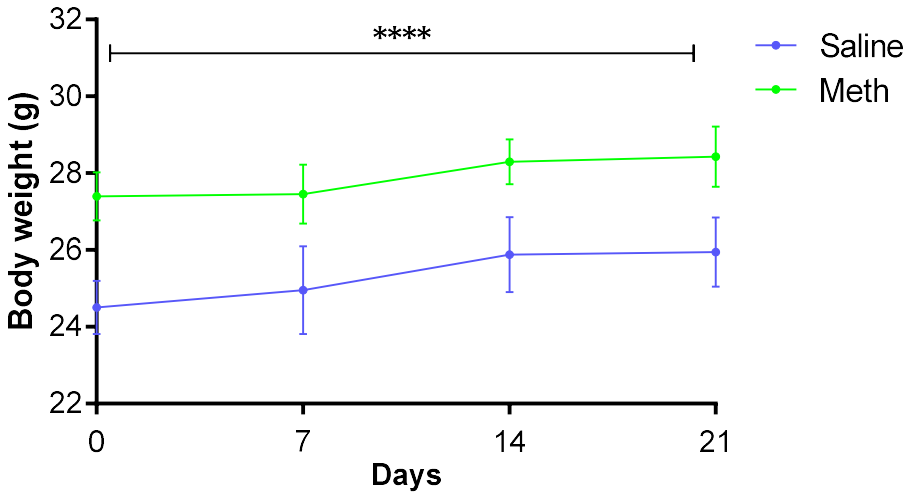


Figure 28 Evolution of body weight of animals from the groups over the total duration of the experiment (21 days); Meth n=4; Saline n=4. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; *p<0.0001

4.9. Emotional fingerprint of METH intoxicated mice 2 weeks after 3 high daily doses of METH

Open Field test

The animals did not show any statistically significant changes in total distance travelled and in mean speed in the open field test , three weeks following METH administration (Figure 29A, B). However, METH mice spent more time immobile than the saline group (Figure 30 A). Nonetheless, there were no statistically significant differences in the number of observed episodes between groups (Figure 30B).

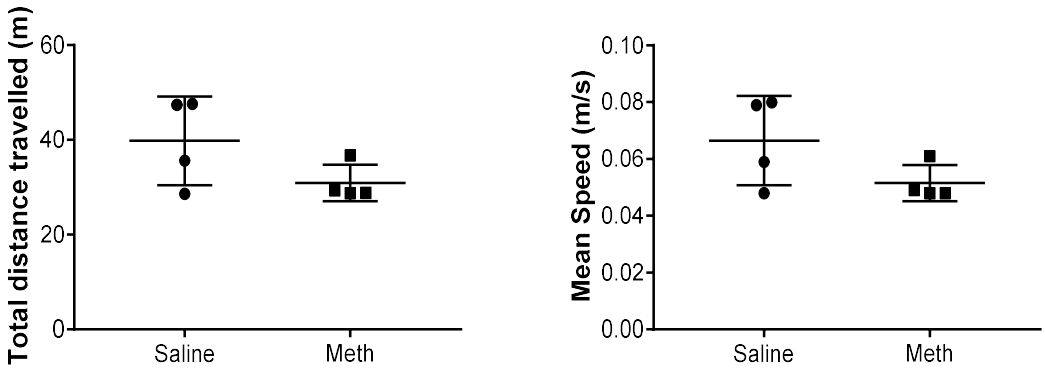


Figure 29. Behavioral analysis in week 3 in a Square Open Field. A: Total Distance travelled (m); B: Total Mean speed (m/s). Sal, METH (n=4). Data are presented in mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

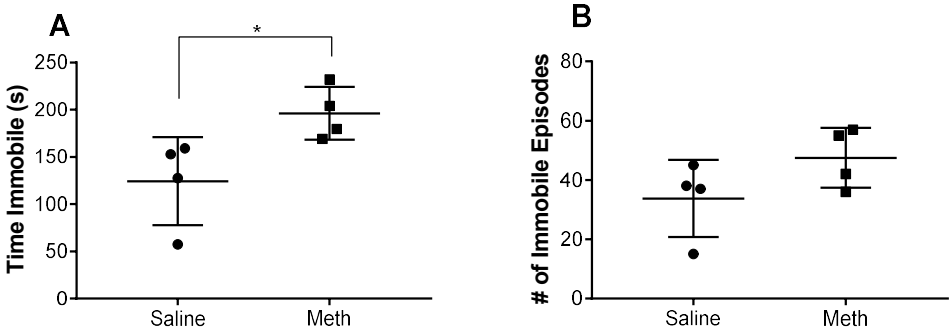


Figure 30 Behavioral analysis in week 3 in a Square Open Field. A: Total immobility time (s); B: Number of immobile episodes. Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; *p<0.05.

The time spent and the distance travelled in center as well as the percentage of distance travelled in the center showed no statistically significant differences between the saline and the METH groups (Figure 31A-C).

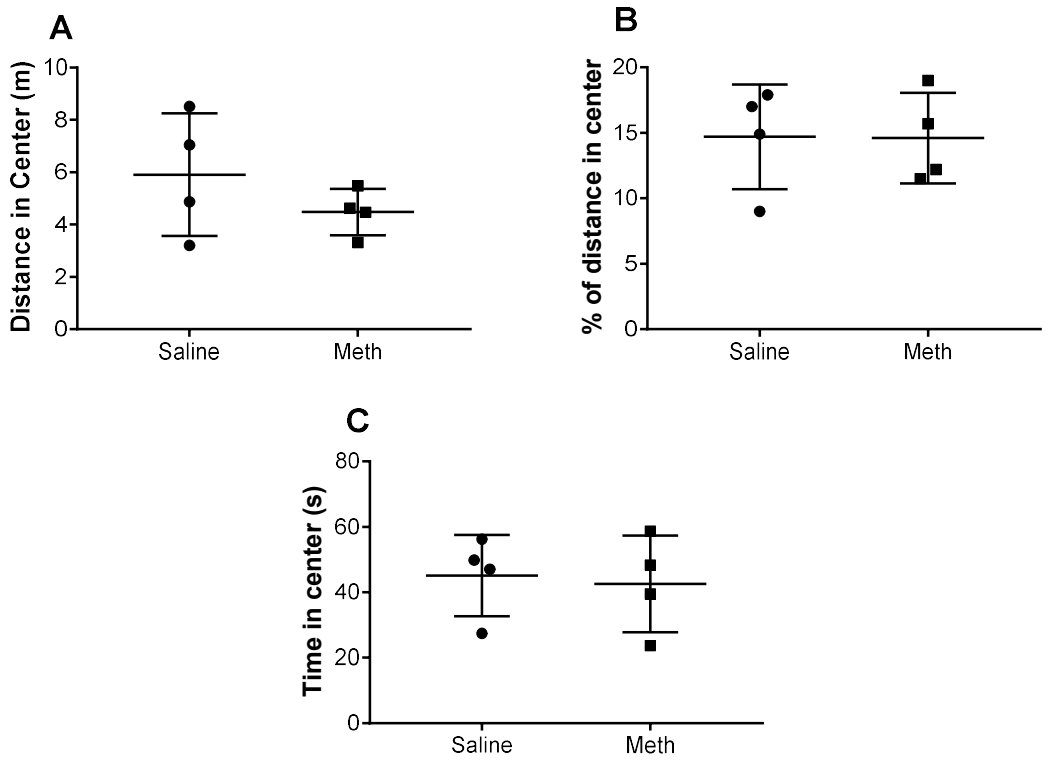


Figure 31. Behavioral analysis in week 3 in a Square Open Field. A: Distance travelled in center; B: Percentage of total distance travelled in center; C: Time spent in center. Sal, METH (n=4). Data are presented in mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Tail Suspension test

The analysis of the tail suspension test revealed no statistically significant differences in the latency to immobility and in the total immobility time between METH and SAL groups (Figure 32A, B).

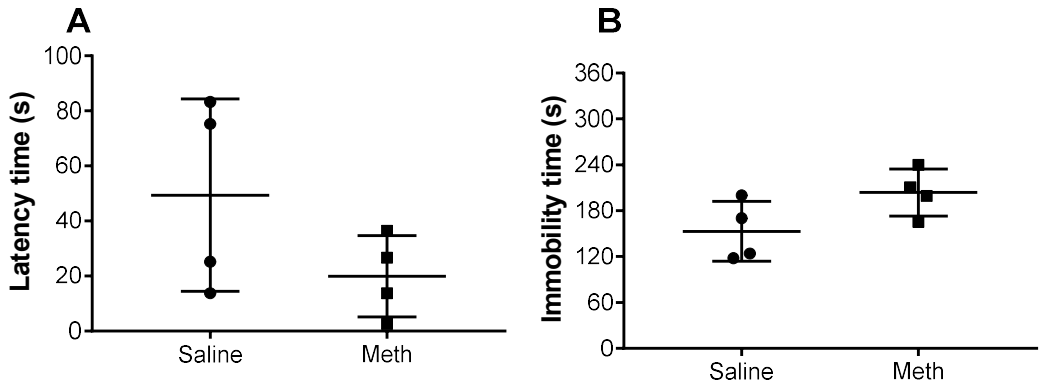


Figure 32. Behavioral analysis in week 3 using Tail Suspension test. **A**: Latency to immobility (s); **B**: Total time spent immobile (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

Splash Test

Latency to dorsal grooming and total grooming time were measured in the Splash test. METH administration showed any significant impact in both (Figure 33A, B).

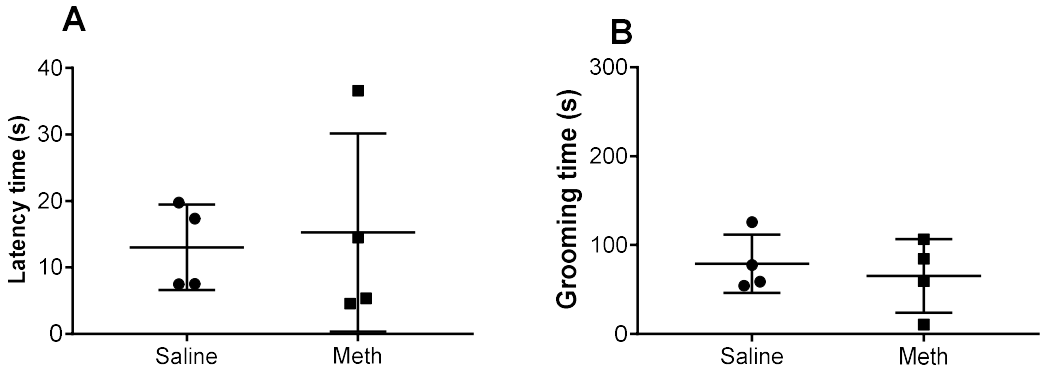


Figure 33. Behavioral analysis in week 3 using Splash test. A: Latency time to dorsal grooming (s); B: Total grooming time (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

Forced Swim test

METH did not significantly change the latency to immobility and the total immobility time in the forced swim test (Figure 34A, B).

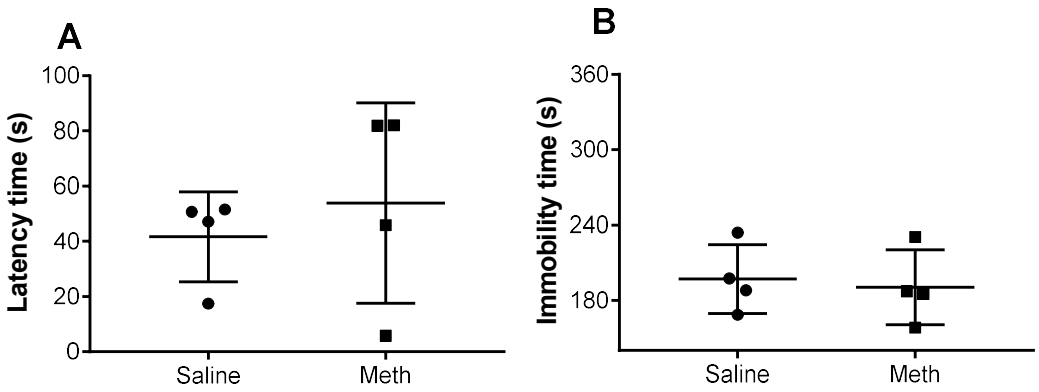
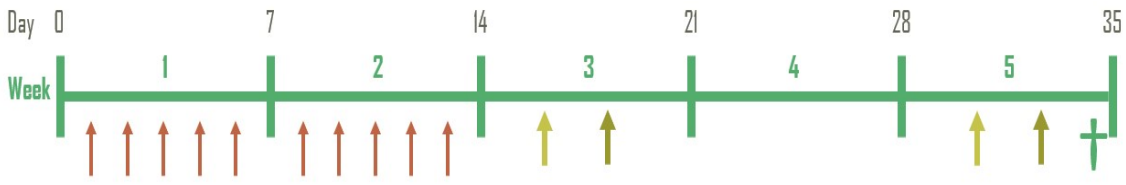


Figure 34. Behavioral analysis in week 3 using Forced Swim test. A: Latency time to immobility (s); B: Total immobility time (s). Sal, METH (n=4). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

4.10. Experimental design IV

The experimental design IV was developed to test the effects of a sub-chronic METH consumption in mice emotional behavior. Animals were tested 4 days and in the third week after METH paradigm treatment. This 2-week window could further prove to be instrumental for a therapeutic intervention.



Scheme 8. Experimental protocol IV: Orange arrows: Meth administration (daily i.p. 5mg/kg in the first week and 10mg/kg in the second week); Light green arrows: Behavioral tests; Green cross: Euthanasia.

Body Weight

The weight of the animals in this protocol suffered no observable change due to the continuous METH administration, occurring only normal weight gains from the passage of time (Figure 35).

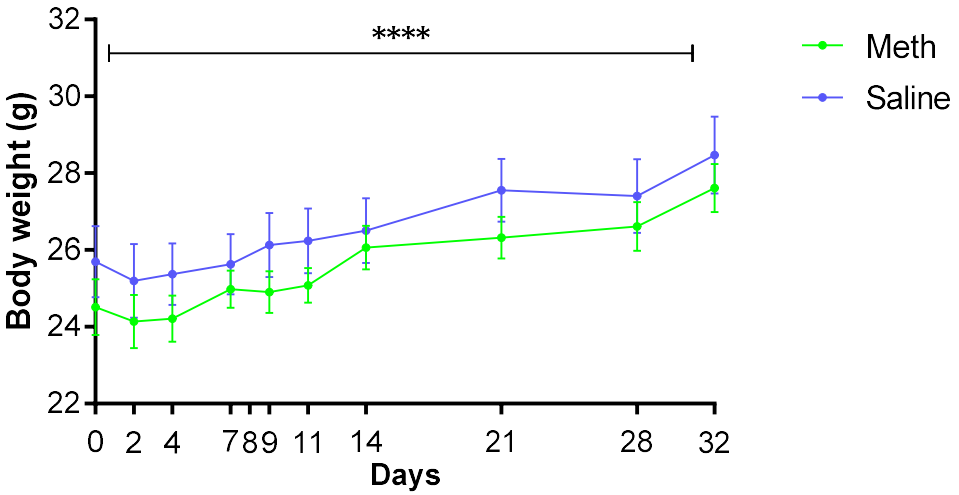


Figure 35 Evolution of body weight of animals from the groups over the total duration of the experiment (21 days); Meth n=8; Saline n=6. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test; *p<0.0001

4.11. Emotional fingerprint of METH-intoxicated mice at day 4 post-METH sub-chronic administration protocol

Open field test

A sub-chronic METH paradigm did not statistically change the locomotor parameters evaluated in the Open Field at 4 days post-METH treatment when compared with saline mice (total distance travelled, mean speed and time of

immobility and number of immobility events; Figure 36A, B and Figure 37A, B, respectively).

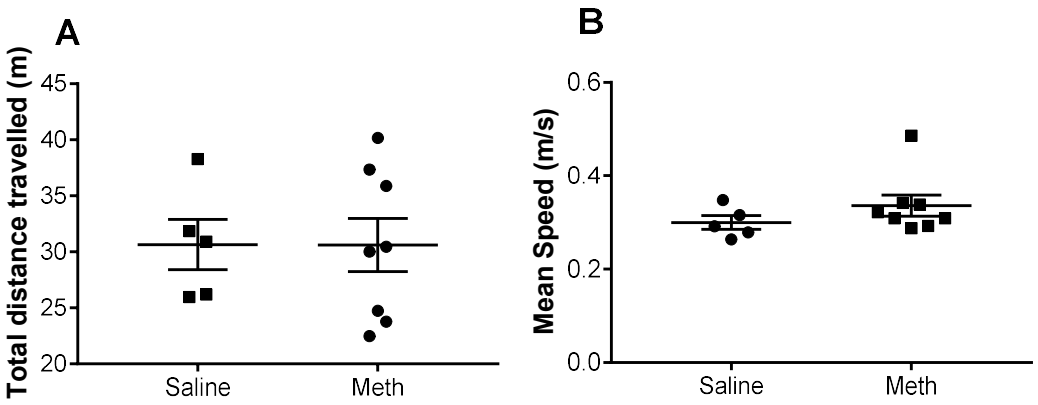


Figure 36. Behavioral analysis at day 4 in a Square Open Field. A: Total Distance travelled (m); B: Total Mean speed (m/s). Sal, METH (n=5-8). Data are presented in mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

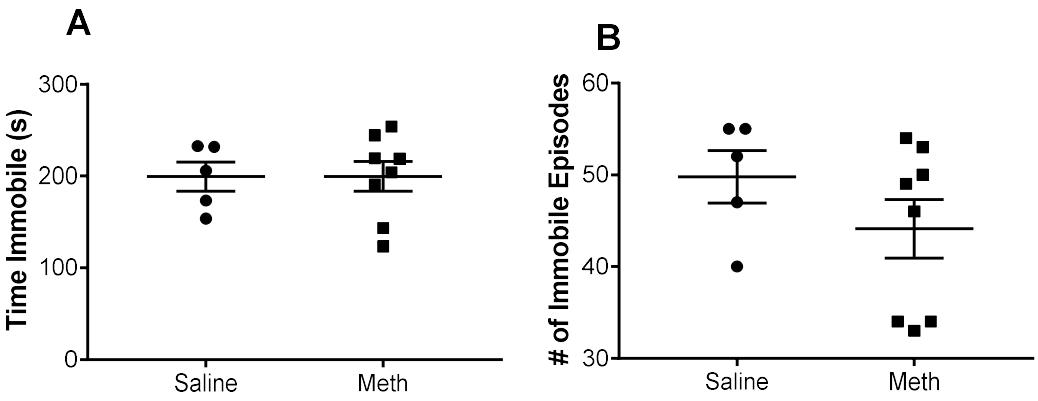


Figure 37 Behavioral analysis on day 4 in a Square Open Field. A: Total immobility time (s); B: Number of immobile episodes. Sal, METH (n=5-8). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.[i]

In the anxiety-related parameters, this experimental design showed no statistically significant differences between groups in the distance travelled in the center (Figure 38A), in the percentage of total distance travelled in center (Figure 38B) and in the time spent in the center (Figure 38C).

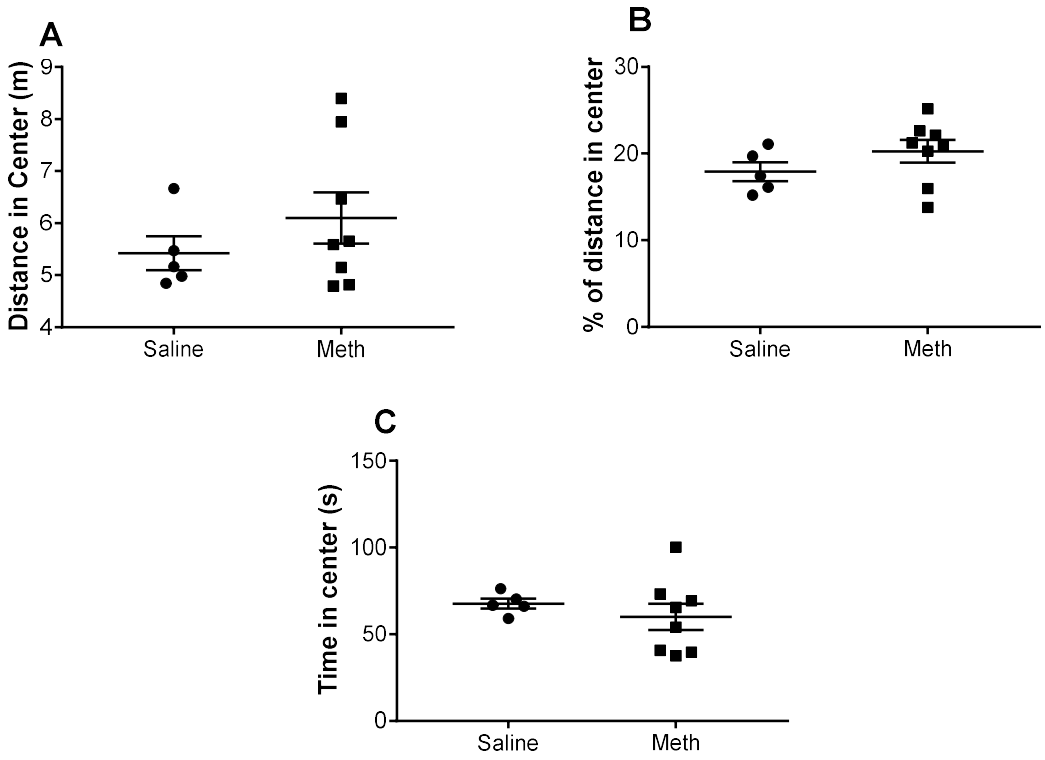


Figure 38. Behavioral analysis at day 4 in a Square Open Field. A: Distance travelled in center; B: Percentage of total distance travelled in center; C: Time spent in center. Sal, METH (n=5-8). Data are presented in mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Tail suspension test

In the tail suspension test both measured parameters (latency time and time immobile) showed no statistically significant differences between the experimental groups (Figure 39A,B).

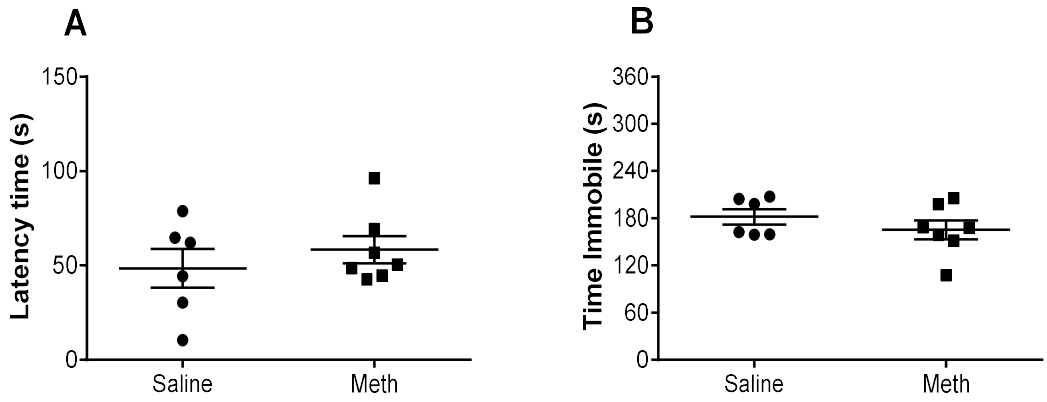


Figure 39. Behavioral analysis on day 4 using Tail Suspension test. A: Latency to immobility (s); B: Total time spent immobile (s). Sal, METH (n=6-7). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Splash test

The behavior of the METH-mice was not statistically different from controls in the splash test (Figure 40A, B).

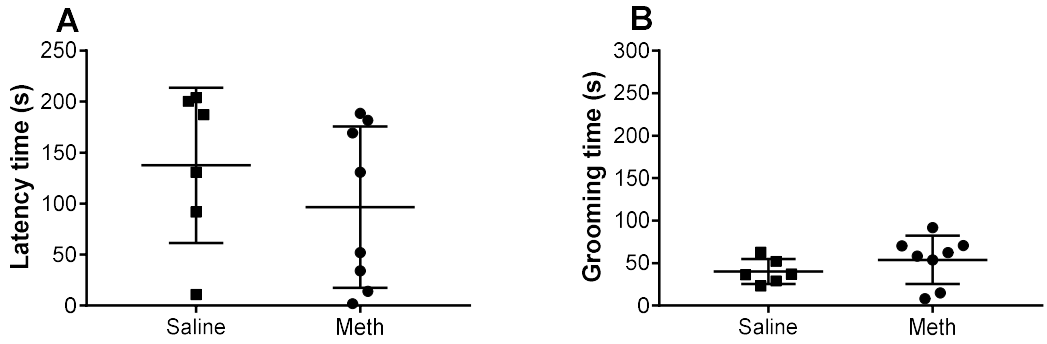


Figure 40. Behavioral analysis on day 4 using Splash test. A: Latency time to dorsal grooming (s); B: Total grooming time (s). Sal, METH (n=6-8). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Forced swim test

The mice exposed to METH did not show statistically significant differences in latency and immobility times when compared with controls (Figure 41A, B).

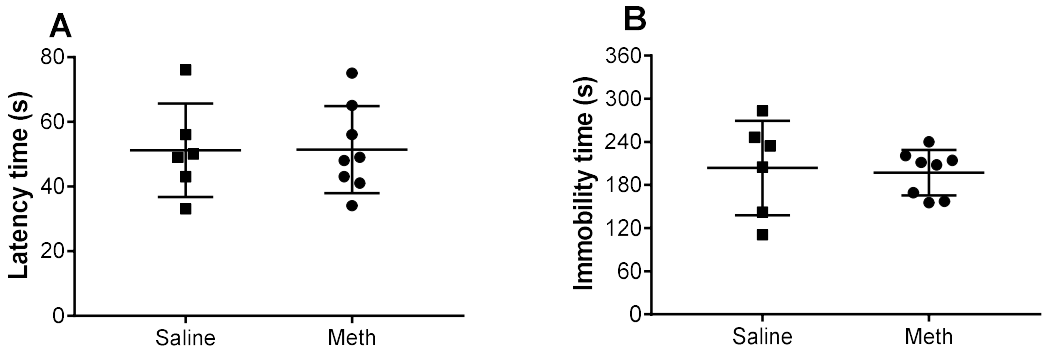


Figure 41. Behavioral analysis on day 4 using Forced Swim test. A: Latency time to immobility (s); B: Total immobility time (s). Sal, METH (n=6-8). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

4.12. Emotional fingerprint of METH-intoxicated mice at three weeks post-METH sub-chronic administration protocol

Open field test

The Open field test was not performed in these animals at this time point, due to the possible carry-over effect between tasks.

Tail suspension test

The animals still did not show any significant differences in the tail suspension test when compared with controls, in the 3rd week post-METH administration (Figure 42A, B).

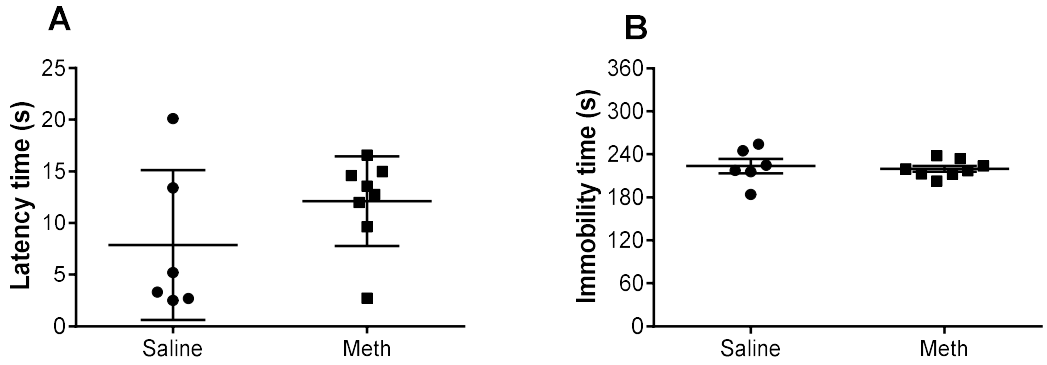


Figure 42. Behavioral analysis on the third week post-METH using Tail Suspension test. **A**: Latency to immobility (s); **B**: Total time spent immobile (s). Sal, METH (n=6-8). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; $p>0.05$.

Splash test

The animals also did not show any significant differences in the splash test when compared with controls, in the 3rd week post-METH administration (Figure 43A, B).

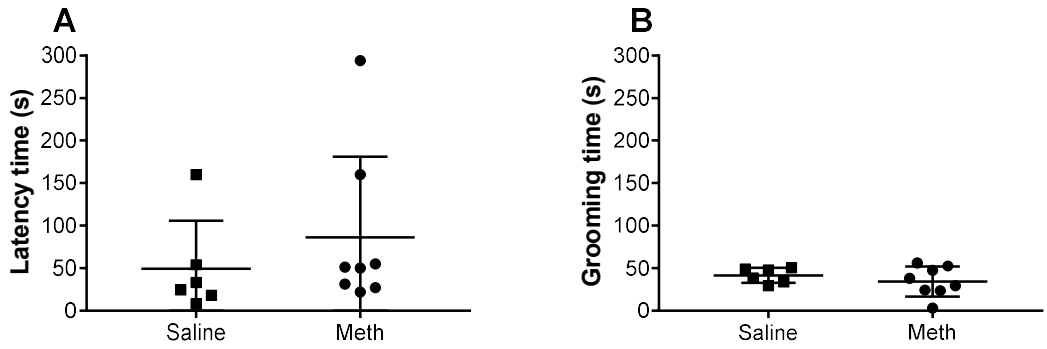


Figure 43. Behavioral analysis on the third week post-METH using Splash test. A: Latency time to dorsal grooming (s); B: Total grooming time (s). Sal, METH (n=6-8). Data are

presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

Forced swim test

Finally, no differences between groups were registered in the 3rd week post- METH regarding the forced swim test results (Figure 44A, B).

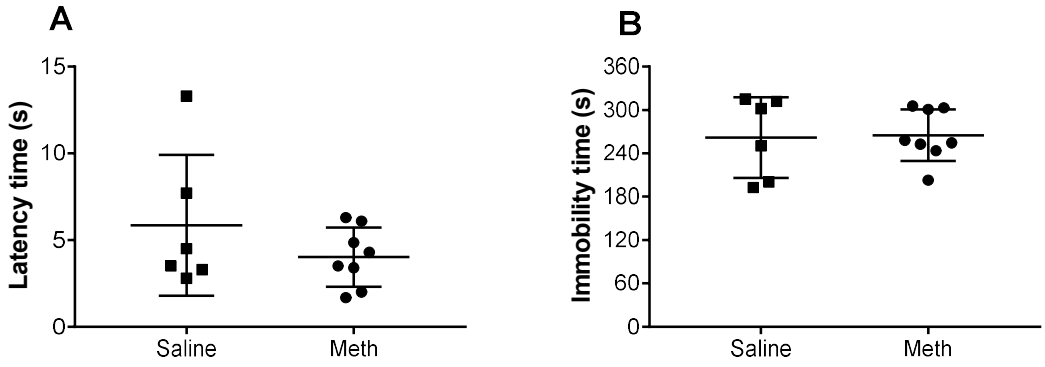


Figure 44. Behavioral analysis on the third week post-METH using Forced Swim test. **A**: Latency time to immobility (s); **B**: Total immobility time (s). Sal, METH (n=6-8). Data are presented as mean ± SD and were analyzed by an unpaired, two-tailed Student´s t-test; p>0.05.

5. Discussion

Animal models have been thoroughly used to study various diseases, including anxiety and depression-related pathologies (Harro, 2018). In this work we used a battery of tests to evaluate locomotor and emotional parameters: open field test, tail suspension test, splash test and the forced swim test. When in the open field apparatus, the animals were evaluated in their locomotor activity, such as the total distance travelled, the mean speed, immobility time and number of immobility episodes during the 10 minutes of the test. This evaluation was to demonstrate that both METH and/or physical exercise had no impact on general activity of mice, which could impair the quality of the data retrieved from the other tests, for example the tail suspension and the forced swim test.

Time spent in center, distance travelled in center and percentage of total distance travelled in center were the parameters analyzed in the open field test, to assess anxiety-like behavior. The time animals spend in center and the distanced travelled in center can give a good representation of the emotional status of the animals. Mice are not from the top of the food chain and they have a lot of predators, therefore their normal instinct is to probably seek protection as they perceive open areas as dangerous, which in this case is the corners/periphery of the open field apparatus, as opposed to the center of the apparatus which is an clear example of an open field and ample space(Seibenhener and Wooten, 2015). On the other hand, mice are also known for being very curious animals and to have a strong exploratory sense, in this manner the anxious and the exploratory traits of the mice clash and in face of novelty (the apparatus itself can be considered a novelty factor to the mice and thus trigger the exploratory side of the animal)(Kulesskaya and Voikar, 2014; Harro, 2018)

The tail suspension test is based on the ability of the mice to escape an aversive and apparently inescapable situation (Yan *et al.*, 2010). In this context, mice have to try to escape using their own movements, as their own tails are taped to a surface which is 50cm above a solid surface. Each animal will sway from immobility to mobility as it struggles to release itself. In this test the despair behavior reads as a depressive-like behavior(Sang *et al.*, 2014). The Forced swim test was used analogously to the Tail suspension test as it also measures the

despair behavior that is associated with a depressive like behavior (Yan *et al.*, 2010; Sang *et al.*, 2014). The forced swim test is a very stressful test to the animals because water is aversive to mice. In this test one forces mice to be in a container full of water at room temperature. The containers used typically do not allow animals to float comfortably as their diameters are small, thus forcing animals to adopt a vertical position without touching the bottom of the container with their tail. In this vertical position in water, animals are forced to swim to be able to float during the duration of the test (6 min).

The splash test is used to measure the grooming behavior, which is associated with the self-care of mice(Isingrini *et al.*, 2010). Mice tend to be very prone to self-care as they groom themselves regularly to remove impurities and debris from its own coat. They are also sometimes grooming their partners, as we watched them in their cages doing so. A lower self-care propensity is linked with a depressive-like behavior as the animal lacks the motivation to clean itself (Isingrini *et al.*, 2010). By splashing the lower back of mice with a sucrose solution, one promotes the grooming of that specific area as well other areas including head and nose. The sucrose solution is used because the probability of capturing natural grooming in a 5-minute interval (duration of the test) is very low and doesn't provide a good baseline for data analysis (Isingrini *et al.*, 2010; Hu *et al.*, 2017)

Firstly, we used herein a neurotoxic dose of METH that induced a long-lasting increase in the immobility time in the tail suspension test (Silva *et al.*, 2014). In fact, these authors showed that control animals spent circa 100 seconds immobile while METH intoxicated mice spent circa 140 seconds immobile 49 days post-METH injection. However, now we failed to reproduce this behavioral profile: both controls and METH-mice spent circa 190 seconds immobile in the tail suspension test. Moreover, other behavioral tests including splash, forced swim and open field tests failed to unveil a long-lasting disturbed emotional state in METH-intoxicated mice. However, one should stress that METH-mice showed a tendency to have a lower weight-gain throughout the 8 weeks-protocol when compared with saline mice. One cannot exclude that basal emotional status of mice prevented one to see further emotional changes using this METH stimuli.

Secondly, we also tested the impact of a physical exercise protocol on mice emotionality. We showed herein that the METH administration prior (4 days) to the

ergoespirometric test had no influence on the Vertical Work, and on the Exhaustion time of the animals. This is suggestive that robust neurochemical perturbations evoked by METH (Silva et al. 2014; Fonseca et al. 2017) administration are not sufficient to impair these physical activity parameters of mice.

On the other hand, the physical protocol implemented in Experimental protocol I with the 15% weekly increase in the overload over the 7 subsequently weeks, revealed itself to be a heavy load to the animals, since some animals ended the protocol running at 264% of the potency, they ran in the Ergoespirometric test. In the last weeks, most animals started failing to comply with the treadmill protocol designed for them, showing signs of exhaustion, like stopping, leading to an increase in the number of strokes per session so the animal would start running again. During the treadmill protocol the electrical grids were not used so the stress induced in the animals was minimized to the maximum (one used gentle strokes and pokes to make the animal running again). However, METH-mice showed a tendency to have a lower vertical work each week when compared to saline mice. One should bear in mind that body weight influences vertical work (please see section 3.8 Treadmill Protocol, page 20). Therefore, this apparent differences between vertical works may reflect the tendency of smaller increments in body weight in METH mice. Finally, this PE protocol did not seem to alter mice locomotor and emotional parameters evaluated by open field, splash, tail suspension and by forced swim tests at 4 and 8 weeks. Moreover, this PE protocol also did not change mice body weight and food intake evolution when compared with sedentary mice. Corticosterone and other physiological parameters are being analyzed including heart and spleen mass to further characterize the physical exercise protocol used herein.

Since METH-mice from the experimental protocol I did not show any evident signs of depressive- and anxiety-like behavior, we tried the Experimental protocol II, where the animals would not have to be handled so often as the animals subjected to physical exercise protocol were. In fact, we hypothesized that the excessive handling of the control groups in the Experimental protocol I, such as being placed in the treadmill in free roam and introduced to novelty and stress-relieving factors, would attenuate the putative emotional impairment imposed by METH. However, the animals from the Experimental protocol II also failed to show

any evident anxious- or depressive-like symptoms in the behavioral tests in week 4 post-METH. However, if anything, METH-mice showed a tendency to spend less time immobile in the tail suspension test. This should be further looked at earlier time points, using more animals in each experimental group.

The rational approach for the Experimental protocol III was that a single neurotoxic dose of METH could not be enough to provoke a long-lasting anxiety- and depressive-like behaviors. Therefore, we injected mice with METH, daily for 3 consecutive days, using the 30mg/kg dose. The injections were given with 24h of interval to avoid overdosing the animals and to mimic a binge-like METH consumption, which is likely observed in real life, for example among music festival goers (Foppe, Hammond-Weinberger and Subedi, 2018). The experimental time window was also shortened (from 4 to 3 weeks), hypothesizing that the 4 weeks (the 8 weeks would be even worse) would be a timeline too long to preserve the emotional alterations purportedly triggered by METH. Interestingly, animals injected with METH showed a significant increase in the immobile time in the open field test. However, it is suggestive that this METH dosing exerts a long-lasting alteration in mice locomotor activity. Nonetheless, other behavioral tests did not show any evident alteration in the emotional mice profile. These observations have to be confirmed using more animals in each experimental condition.

In the Experimental protocol IV, we adopted a sub-chronic administration of METH, with an increase in the dosage at the 2nd week of the protocol. This was done to mimic as accurately as possible the real life of METH users, who start with a recreational dose but as time passes by and that same dose starts failing to provide the expected “high” (because of the mechanisms underlying the addiction), METH users start to increase the dosages they intake (Kitamura *et al.*, 2006).

From one week to another with a 2-day interval (weekend) the dose was doubled: from 5mg/kg to 10 mg/kg. Every time the METH was administered the animals showed clear signs of METH intoxication (including agitation, piloerection and a change in ear positioning). However, we did not find any statistically significant changes between groups in both studied time-points. These results seem to be in contrast with Ren et al. 2015 that showed that administration of 3 mg/kg s.c continuously for 5 days increased immobility time of C56Bl6 mice in TST and in FST up to 14 days post-last METH administration. It is unlikely that a higher

METH dose for longer time (one more week) used herein might have attenuated the negative emotional impact of METH. Further work needs to be done to clarify the impact of METH on depressive- and anxiety-like behavior in mice.

6. Conclusion

In this study it was not possible to find the emotional fingerprint of METH intoxicated animals. However, we and others have been suggesting that METH administration is a pharmacological inducer of depressive-like behavior in mice. Currently the most used inducer of a long-lasting depressive-like behavior is the Chronic Unpredictable Mild Stress (Antoniuk *et al.*, 2019). Nonetheless, this is a very troublesome and time-consuming protocol. In the future it would be interesting to further explore the strategy used in Experimental protocol III. In fact, achieving a quick and reliable pharmacological method to induce negative emotionality (anxiety- and depressive-like behavior) in mice to substitute the current available methods could impact the amount of research in the field. Finally, anxiety and depression tend to become very common in the life of the people as the world is continuing to evolve, so more research on the topic eventually leads to a development of better strategies to overcome these pathologies and to improve the world's public health status.

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