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Impaired frontal cortical energy metabolism underlies an anhedonic-like behaviour in METH-treated mice

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

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Index

List of tables

List of figures

Abbreviations

^{1}H-NMR - Proton Nuclear Magnetic Ressonance

18F-FDG PET - Fluorine-18-fluorodeoxyglucose Positron Emission Tomography

2DG - 2-[^{14}C]deoxyglucose

ADHD - Attention-Deficit/Hyperactivity Disorder

AMPH - d-amphetamine

ANLS - Astrocytic-Neuronal Lactate Shuttle

ATP - Adenosine Triphosphate

ATS - Amphetamine-type Stimulants

BCA - Bicinchoninic acid assay

BGM - Brain Glucose Metabolism

CBF - Cerebral Blood Flow

CNS - Central Nervous System

CO_2 - Carbone dioxide

CPMG - Carr–Purcell–Meiboom–Gill

DOC - Sodium deoxycholate

DTT - Dithiothreitol

ECF - Enhanced Chemifluorescence Substrate

EPM - Elevated-Plus Maze

EAAT's - Excitatory Aminoacid Transporters

FDG - [F-18]fluorodeoxyglucose

FMUC - Faculty of Medicine, University of Coimbra

Fructose-6P - Fructose-6-phosphate

FST - Forced-swim test

GLN - Glutamine

GLU - Glutamate

GluT's - Sodium-independent Glucose Transporters

hBEC - human Brain Endothelial Cell

HK - Hexokinase

HRMAS - High Resolution Magic Angle Spinning

LDH - Lactate Dehydrogenase

LC - *locus coeruleus*

MDD - Major Depressive Disorder

MDMA - 3,4-methylenedioxy-*N*-methylamphetamine

METH - *N*-methyl-*O*-phenyllisopropylamine

MCT's - Monocarboxylate Transporters

NAA - N-acetyl-aspartate

NADH - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

OF - Open-field

PET - Positron Emission Tomography

PFC - Prefrontal Cortex

PMSF - Phenylmethylsulfonyl Fluoride

PPP - Pentose Phosphate Pathway

PSNS - Peripheral Sympathetic Nervous System

PVDF - Polyvinylidene Fluoride

rCMRglc - Relative Regional Cerebral Glucose Metabolism

SAL - Saline Group

SNAP-25 - Synaptosome-associated Protein

SDS - Sodium Dodecyl Sulfate

TCA - Tricarboxylic Acid

WB - Western Blot

Abstract

Methamphetamine (METH) is an illegal stimulant drug with over 500,000 individuals estimated to abuse METH, in the United States, each month (Substance Abuse and Mental Health Services Administration, 2010). Long term users are at greatly increased risk for psychiatric problems including depression pathology (Curtis 2006; Hendrickson et al. 2008; Kaye et al. 2007; Zweben et al. 2004). The present study aims to characterize the effect of a single-high neurotoxic METH dose (30mg/kg) on early mood behaviour and on the underlying frontal cortical and hippocampal metabolic profile in mice. Three days post-METH injection mice showed an anhedonic state and decreased self-care, with a rather diminished self-grooming time, in the splash test (p=0,0348). This confirms a previously reported depressive-like behavior. However, elevated plus maze excluded anxiety-like behavior in METH-intoxicated mice. A ^{1}H-NMR metabonomics analysis showed a decrease in both NAA/creatine and lactate/alanine ratio in frontal cortical from METH-intoxicated mice, 3 days post-treatment. This is suggestive that METH imposed disturbed frontal cortical energetics. In spite of metabolic alterations the METH-intoxicated mice failed to show synaptoxicity as probed by normal synaptophysin and Syntaxin 1 levels when compared with control animals. However the metabonomics analysis showed that other parameters including astrocytic, glutamatergic and gabaergic markers were similar to control animals. Additionally, the hippocampal metabolic profile from METH-intoxicated mice was not statistically different from control mice. Herein, we provide evidence, for the first time, that anhedonic-like behavior is underlined by a frontal-cortical perturbed energy metabolism.

Resumo

A Metanfetamina é uma substancia psicostimulante viciante, amplamente consumida em todo o mundo, constituindo um grave problema para a saúde pública mundial. Uso abusivo desta droga aumenta o risco de desenvolvimentos de problemas psiquiátricos, como a depressão.

O presente estudo tem como objetivo a caracterização do efeito de uma única dose neurotóxica de METH (30mg/kg) no comportamento de ratinhos, bem como compreender as alterações metabólicas no córtex e hipocampo associadas a tal administração. Três dias após a injeção de METH, os animais revelaram anedonia e baixo *self-care*, com diminuição significativa do tempo de *grooming* no teste *splash* (p=0,0348). Tal resultado confirma o comportamento tipo depressivo anteriormente descrito. Por outro lado, o *elevated plus maze* excluiu comportamento tipo ansioso associado à exposição à METH.

A análise por Ressonância Magnética Nuclear exibe diminuições nos níveis de NAA/creatina e na razão lactato/alanina nos córtices pré-frontais dos animais do grupo METH, três dias após o tratamento. Tal informação é sugestiva do impacto da METH no metabolismo energético desta região cerebral. Apesar das alterações metabólicas, níveis normais de sinaptofisina e sintaxina revelaram ausência de sinapto-toxicidade associada à intoxicação por METH. Outros parâmetros, incluindo marcadores astrocíticos, glutamatérgicos e gabaergicos registaram valores semelhantes aos controlos, bem como os níveis metabólicos no hipocampo, que também sofreram não alterações.

Assim, provamos pela primeira vez que o estado de anedonia é associado a perturbações no metabolismo energético no córtex pré-frontal.

Chapter 1

Introduction

1. Brain Glucose Metabolism

Unlike other organs, the energy supply of the brain is almost exclusively by aerobic glucose degradation. In the adult brain, neurons have the highest energy demand, and, since its capacity to store energy is extremely limited, it requires continuous delivery of glucose from blood. In humans, the brain account for approximately 2% of the body weight, but consumes approximately 20% of glucose-derived energy, making it the main consumer of glucose (approximately 5.6 mg glucose per 100 g human brain tissue, per minute) (Berg et al., 2002).

Glucose metabolism provides the fuel for physiological brain function through the generation of ATP, the foundation for neuronal and non-neuronal cellular maintenance, as well as the generation of neurotransmitters. As such, substrate selection by the brain is highly specific, while peripheral organs can metabolize glucose, fat or proteins. Fatty acids cannot traverse the blood-brain barrier (Peters et al., 2004).

Therefore, regulation of glucose metabolism is critical for brain physiology and disturbed glucose metabolism in the brain underlies several diseases affecting both the brain itself, as well as the entire organism - obesity, depression, polycystic ovaries and metabolic syndrome, among others (Mergenthaler et al., 2013).

1.1 Glucose metabolic pathways

In astrocytes, glucose is driven across the endothelial membranes of the blood-barrier barrier, via glucose transporter 1 ($GluT_1$). This transporter has two isoforms with different abundance in different cellular types: the 55kDa isoform is more abundant in the endothelial cells, whereas the 45kDa-form is expressed in astrocytes (Figure 1). $GluT_1$ further mediates glucose uptake from extracellular fluid into astrocytes,

oligodendroglia, and microglia, while $GluT_3$ facilitates neuronal glucose uptake. Glucose transport capacity exceeds demand over a wide range, and the higher transport rate of $GluT_3$ ensures that neurons have sufficient glucose supplies under varying glucose levels and different activity states (Breu, Guggenbichler, & Wollmann, 2008a). Only traces of other GluT isoforms are found in the brain; of these, $GluT_4$ has been detected in distinct neuronal populations and $GluT_5$ in microglia.

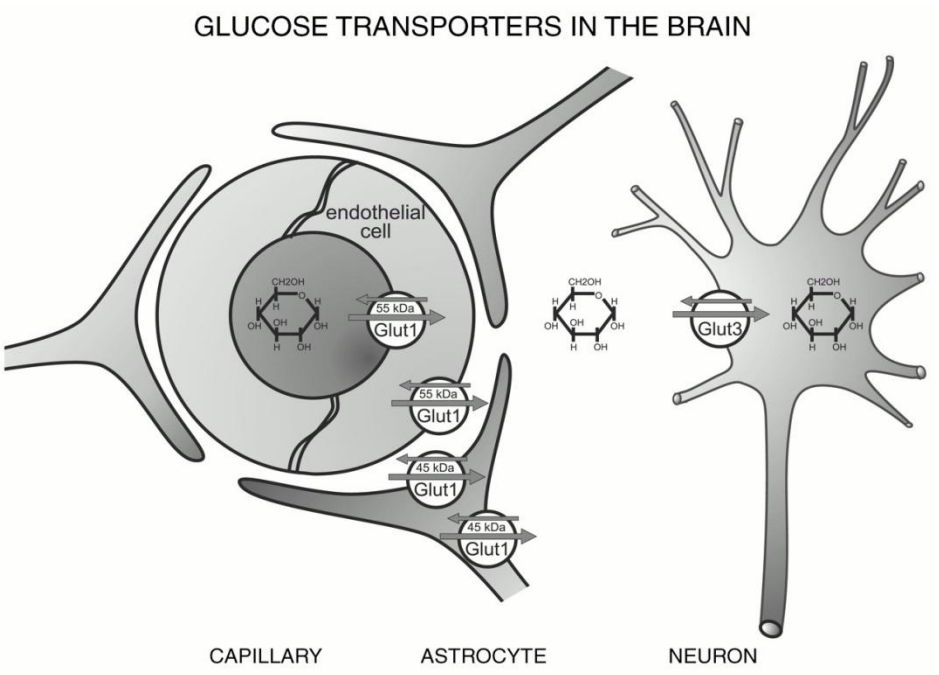

Figure 1 - Location of different glucose transporter isoforms in the brain. $GluT_1$ 55-kDa isoform is abundant in the endothelial cells, whereas the 45-kDa isoform is expressed in astrocytes. $GluT_3$ is the neuronal glucose transporter. Only traces of other GluT isoforms are found in the brain; of these, $GluT_4$ has been detected in distinct neuronal populations and $GluT_5$ in microglia. From: Duelli and Kuschinsky, Physiology, 2001.

Once inside brain cells, glucose is phosphorylated by hexokinase (HK) to Glucose-6-phosphate (G-6-P), which can follow three different metabolic pathways. Firstly, G-6-P can be metabolized through the Pentose Phosphate Pathway, producing reducing equivalent in the form of NADPH. Secondly, G-6-P can follow glycogenesis, enriching glycogen stores. Finally, it can give rise to two molecules of pyruvate, plus ATP and NADH, through glycolysis. Pyruvate can enter the mitochondria to be metabolized through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, thus originating ATP and CO_2. Alternatively, in astrocytes, Lactate Dehydrogenase (LDH) can reduce pyruvate to lactate, which can be released into the extracellular space through monocarboxylate transporters (MCT's) and taken up by neurons, where it converts back to pyruvate and originates ATP, at the mithocondria. (Bélanger, Allaman, & Magistretti, 2011) (Figure 2).

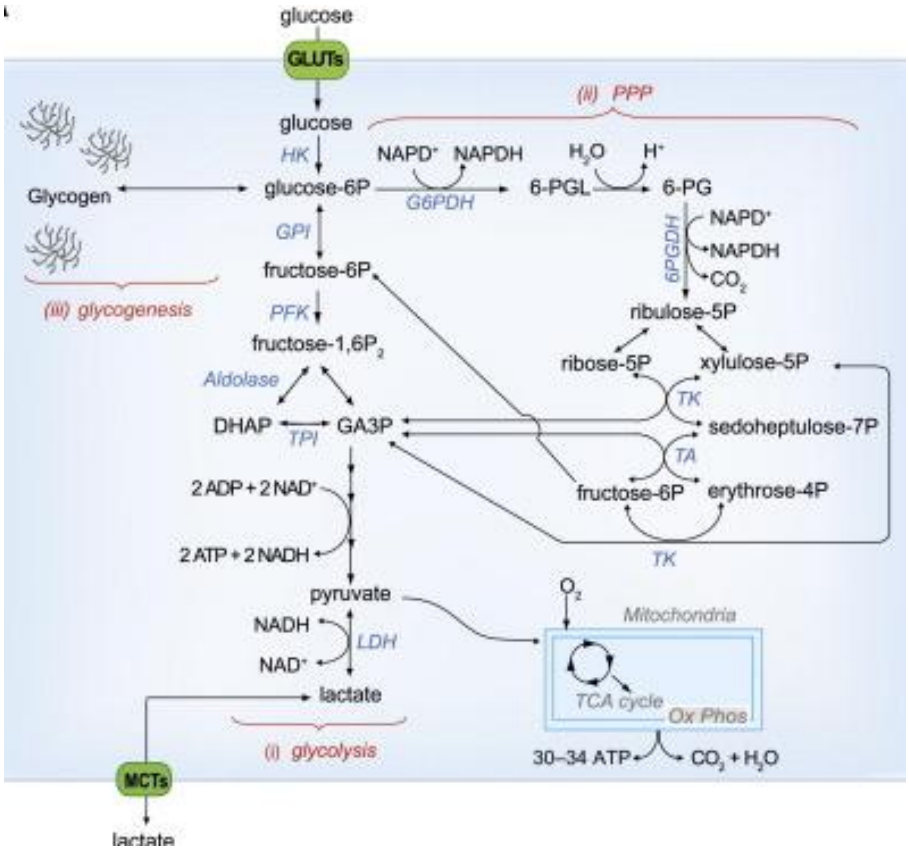

Figure 2 - Brain Glucose Utilization in the brain: Schematic representation of glucose metabolism. Glucose enters cells trough glucose transporters (GluTs) and is phosphorylated by HK to produce glucose-6-phosphate. Glucose-6P can be processed into three main metabolic pathways. First, it can be metabolized through glycolysis (i), giving rise to two molecules of pyruvate and producing ATP and NADH. Pyruvate can then enter mitochondria, where it is metabolized through the TCA cycle and oxidative phosphorylation, producing ATP and CO_2 while consuming oxygen. In astrocytes, pyruvate can otherwise be reduced to lactate by lactate dehydrogenase (LDH) and then released in the extracellular space through monocarboxylate transporters (MCTs). The complete oxidation of glucose produces larger amounts of energy in the form of ATP in the mitochondria (30–34 ATP) compared to glycolysis (2 ATP). Alternatively, glucose-6P can be processed through the pentose phosphate pathway (PPP) (ii), leading to the production of reducing equivalent in the form of NADPH. Note that the PPP and glycolysis are linked at the level of glyceraldehyde-3-phosphate (GA3P) and fructose-6-phosphate (fructose-6P). Finally, in astrocytes, glucose-6P can also be used to store glucosyl

units as glycogen (iii). Abbreviations are as follows: GPI, glucose-6-phosphate isomerase; PFK, phosphofructokinase-1; Fructose-1,6-P2, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; TPI, triose phosphate isomerase; G6PDH, glucose-6-phosphate dehydrogenase; 6-PGL, 6-phosphoglucono-d-lactone; 6-PG, 6-phosphogluconate; 6 PGDH, 6-phosphogluconate dehydrogenase; ribulose-5P, ribulose-5 phosphate; ribose-5P, ribose-5-phosphate; xylulose-5P, xylulose-5-phosphate; TK, transketolase; sedoheptulose-7P, sedoheptulose-7 phosphate; TA, transaldolase; and erythrose-4P, erythrose-4-phosphate. Taken from Bélanger et al., 2011.

1.2 **Glutamate - glutamine cycle**

Glutamate (GLU) is an amino acid neurotransmitter that does not cross the BBB intimately involved in neurotransmission, being strongly dependent on astrocytic metabolism (Hertz & Dienel, 2002). In fact, due to the lack of the enzyme pyruvate carboxylase, neurons depend on astrocytes for *de novo* synthesis of glutamate.

Indeed, GLU is released at the synapse, activates glutamatergic receptor and must be removed rapidly. Neurons remove little GLU from the synapse, being the largest portion of GLU taken up by astroglia, through excitatory aminoacid transporters (EAAT's; mainly $EEAT_1$ and $EEAT_2$) via a sodium-dependent mechanism. (Danbolt, 2001). Once inside, the cycle starts with GLU amidation to glutamine in an ATP-requiring reaction in which an ammonium ion is fixed into GLU. This reaction is catalyzed by glutamine synthase - an enzyme almost exclusively localized in astrocytes - and provides an efficient means of disposing not only of GLU, but also ammonium. Glutamine (GLN) is then released by astrocytes and is taken up by neurons where it is hydrolyzed back to glutamate by glutaminase, thus contributing to the replenishment of the neurotransmitter pool of GLU in neurons (Shen, 2013).

Overall, glutamate enters the astrocytes, via EEAT1, together with 3 Na^+ ions. The astrocytic Na^+/K^+ ATPase responds predominantly to increases in intracellular sodium concentrations and, consequently, astrocytes increase glucose uptake, through $GluT_1$, followed by lactate production and release, through glycolysis (Pellerin & Magistretti, 1994; 2003).

This metabolic pathway, allows the removal of potentially toxic excess of GLU from the extracellular space while returning to the neuron a synaptically inert precursor that will lead to the regeneration of the neuronal pool of GLU (Daikhin & Yudkoff, 2000).

This evidence supports the idea that glutamate uptake-induced aerobic glycolysis in astrocytes serves a neurometabolic coupling between astrocytes and neurons.

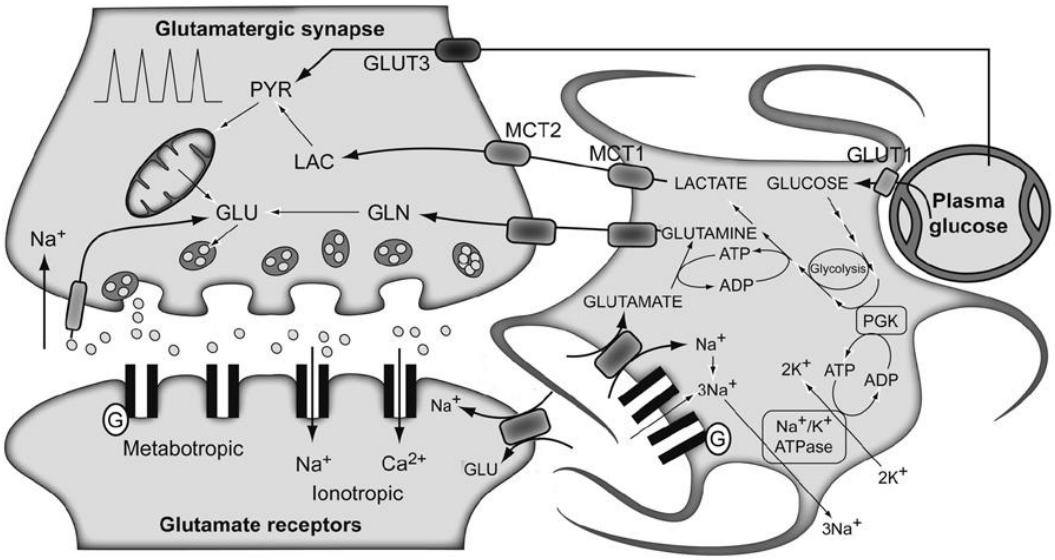

Figure 3 - Glutamate-glutamine cycle and the the metabolic coupling hypothesis between neurons and astrocytes. Glutamate released to the synaptic cleft during glutamatergic neurotransmission is co-transported with Na^+ to the astrocytes. Astrocytic Na^+ is exchanged by extracellular K^+ through the Na^+/K^+ ATPase, consuming one ATP molecule. Astrocytic glutamate produces glutamine through glutamine synthetase, consuming one additional ATP molecule. Lactate that is produced exclusively in astrocytic glycolysis to support these energy demands is extruded to the extracellular medium, taken up by the surrounding neurons and oxidized as their main metabolic fuel. Note the apparent stoichiometric coupling between glutamate–glutamine cycling and glucose uptake as well as the exclusive glycolytic or oxidative metabolisms in astrocytes and neurons, respectively. Gln, glutamine; Glu, glutamate; $GluT_1$ and $GluT_3$, glucose transporters 1 and 3; Lac, lactate; MCT_1 and MCT_2, monocarboxylate transporters 1 and 2; PGK, phosphoglycerate kinase; Pyr, pyruvate. Figure taken from Tsacopoulos&Magistretti,1996.

1.3 **Astrocyte-neuron Lactate Shuttle**

Lactate was first discovered in sour milk by Swedish chemist, Carl Wilhelm Scheele, in 1780. It is produced naturally by the body, for example when muscles are at work. In the brain, it is regarded as an energy source which can be delivered to neurons as fuel to keep them working when brain activity increases (Dienel, 2012). As a product of glycolysis, - which provides the majority of ATP to normal cells - the accumulation of lactate leads to the decrease of the intracellular pH and cessation of glycolysis. In order for glycolysis to continue at a high rate, this molecule must be transported out of the cell, to neurons, where it can be converted to pyruvate, through Lactate dehydrogenase (LHD_1), and enter the TCA cycle, producing ATP, CO_2 and water.

Magistretti and Pellerin (1999) proposed an astrocytic-neuronal lactate shuttle (ANLS) (Figure 4), in which glutamate, released from neurons during synaptic activity, is taken up by astrocytes trough the glutamate-glutamine cycle, activating the Na^+-K^+-ATPase, which triggers glucose uptake, resulting in the rapid formation and release of lactate from astrocytes via monocarboxylate transporters (MCT_1 and MCT_4). The lactate released by astrocytes into the extracellular space is, according to this hypothesis, transported into neurons by MCT_2 and used to fuel activity-dependent energy demands. Additionally, recent studies describe new roles for the lactate in the brain, as a signalling molecule in the *locus coeruleus* (LC). The LC is the principal source of noradrenaline to the frontal brain and thus one of the most influential modulatory centres of the brain. In fact, astrocytes release L-lactate, which excites LC neurons and triggers release of noradrenalin (Tang et al., 2014).

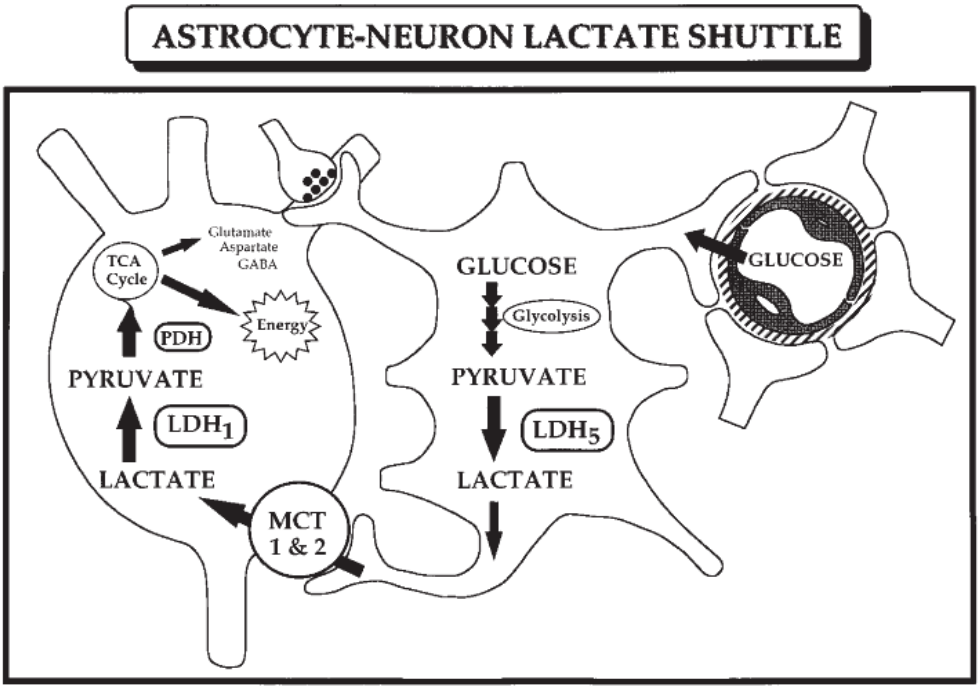

Figure 4 - Schematic representation of proposed astrocyte-neuron lactate shuttle. After neuronal activation and synaptic glutamate release, glutamate reuptake into astrocytes triggers increased glucose uptake from capillaries via activation of an isoform of the Na^+-K^+-ATPase, which is highly sensitive to ouabain (possibly the α_2-isoform). Glucose is then processed glycolytically to lactate by astrocytes that are enriched in the muscle form of LDH (LDH_5). The exchange of lactate between astrocytes and neurons is operated by monocarboxylate transporters (MCT). Lactate is then converted to pyruvate because neurons contain the heart form of LDH (LDH_1). Pyruvate, via the formation of acetyl CoA by pyruvate dehydrogenase (PDH), enters the tricarboxylic acid (TCA) cycle, thus generating 17 ATP/lactate. From: Magistretti and Pellerin (1999).

1.4 Alterations in brain's glucose metabolism relation with psychiatric disorders

As been stated before, astrocytic glucose metabolism plays a crucial role as energy source for brain function. Therefore, a disruption in this pathway may be associated to neurologic and psychiatric disorders as well as to neurotoxicity (Picco et al., 2014).

In fact, previous studies have confirmed this association. Oxidative stress was proven to play a role in impairing brain glucose utilization in elderly subjects with increasing severity of cognitive disturbance. Brain glucose metabolism progressively decreased in the bilateral posterior temporoparietal and cingulate cortices across the three groups: subjects with subjective cognitive impairment, patients with mild cognitive impairment and those with mild Alzheimer's disease.

PET imaging also disclosed that different patterns of brain glucose metabolism were present in epilepsy patients at early stages of the disease. (Nasrallah & Dubroff, 2013).

Metabolic deregulation influences brain function and disturbances in peripheral glucose regulation might be associated with cognitive impairment and depressed mood. Results showed an association between depressive symptoms and glucose metabolism status, but not anxiety (Bouwman et al., 2010).

Drug abuse is also intimately associated to brain glucose metabolism disruption. For example, cocaine shifts glucose metabolism of differently between anatomical areas: the shift in glucose metabolism was greatest in the thalamus when compared to frontal cortex and striatum (Kaplan et al., 2013).

The 2-deoxyglucose method was used to study the effects of acute administration of small intravenous doses of heroin on rates of glucose utilization in rat brain to identify

brain regions that may be involved in the acute behavioral effects of heroin. Administration of 60 μg/kg of heroin, intravenously, resulted in glucose utilization rates that were 16% lower than saline-treated animals (Martin et al., 1997).

The relation between drug abuse, major depressive disorder and glucose metabolism disruption will be further discussed on this work.

2. Drug abuse

The recreational use of illegal drugs, such as cannabis, cocaine and methamphetamine, imposes great human and financial costs on society, due to premature deaths, drug-related crime, health care expenditures and reduced productivity (Justinova, Panlilio, & Goldberg, 2009).

The National Institute on Drug Abuse defines addiction as a chronic, relapsing brain disease, characterized by compulsive drug seeking and use, despite harmful consequences. It is considered a brain disease since it causes long lasting alterations to the brain structure and function and can lead to harmful, often self-destructive, behaviors (http://www.drugabuse.gov).

According to the 2013 European Drug Report, at least 85 million adult Europeans have used an illicit drug at some point in their lives, representing around a quarter of Europe's adult population, whereas over 250.000 drug-related deaths were reported in the USA and more than 250 billion dollars were spent on this matter.

The initial events that lead to addiction involve acute effects at a specific site (or sites) of action of a drug of abuse, as a transporter or receptor. These sites of action typically activate neuronal networks associated with positive reinforcement. Repeated 'on–off' exposure to a drug of abuse progressively leads to stable molecular and cellular changes in neurons, which alter the activity of the corresponding neuronal networks, leading to neurobiological changes in the brain reward circuits and behaviors characteristic of addiction: tolerance, sensitization, dependence, withdrawal and craving (Kreek, LaForge, & Butelman, 2002). The combination of positive (e.g., euphoria) and negative (e.g., withdrawal symptoms) reinforcement may provide a powerful motivational force for compulsive drug taking. Associated neurobiological changes and

behavioral abnormalities and deficits in cognitive function may persist for months or years after discontinuation of drug use (Breu, Guggenbichler & Wollmann, 2008b).

2.1 Amphetamine-type Stimulants (ATS)

Amphetamine (1-Methyl-2-phenylethylamine) gave the name to a group of stimulant compounds, with similar structures and biological properties, called amphetamine type-stimulants (ATS) or 'amphetamines'. This group also includes methamphetamine (METH) and 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) - widely known as ecstasy. – These are all considered popular psycho-stimulant drugs, causing sensations of euphoria and wakefulness.

In the US, amphetamine is a FDA approved treatment for attention-deficit/hyperactivity disorder (ADHD) and narcolepsy, Amphetamines have been used illegally, mainly among young population, since the FDA limited them to prescription use in 1965 (Berman et al., 2008).

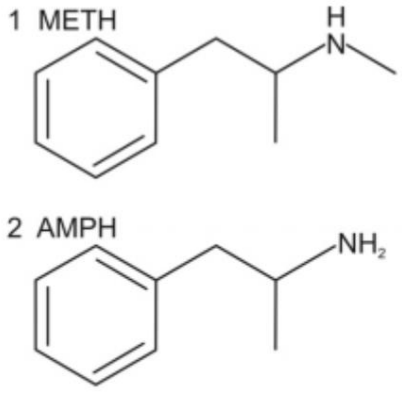

Figure 5 - Chemical structure of methamphetamine (METH) (1), as well as the closely related psychostimulant d-amphetamine (AMPH) (2). Adapted from Barr et al., 2006.

2.2 Epidemiology of abuse of amphetamine-type stimulants

The World Drug Report issued in 2013 reported signs that the market for amphetamine-type stimulants (ATS) is expanding: seizures and consumption levels are increasing, manufacture seems to be spreading and new markets are developing. The use of ATS, excluding "ecstasy", remains widespread globally, and appears to be increasing in most regions. In 2011, an estimated 0.7 per cent of the global population aged 15-64, or 33.8 million people, had used ATS in the preceding year.

The United Nations Office on Drugs and Crime (UNODC) estimated that the worldwide production of ATS is nearly 500 metric tons a year, with 24.7 million abusers, in 2010.

According to the European Drug Report (2013), amphetamine is widely use in Europe, while methamphetamine consumers in Europe are mainly from Czech Republic, where the drug is produced in small illegal laboratories for domestic consumption but also for exportation. In Czech Republic, Sweden, Finland and Slovakia, 20-60% of those seeking drug abuse treatment are due to ATS consumption. In Portugal, the METH consumption is more common among the young population.

Hunt et al. (2007) characterized the target population as being Caucasian, unemployed, single or divorced people who live in rural or suburban areas. As for the gender, unlike many other illegal drugs, methamphetamine is a drug that appeals equally to men and women.

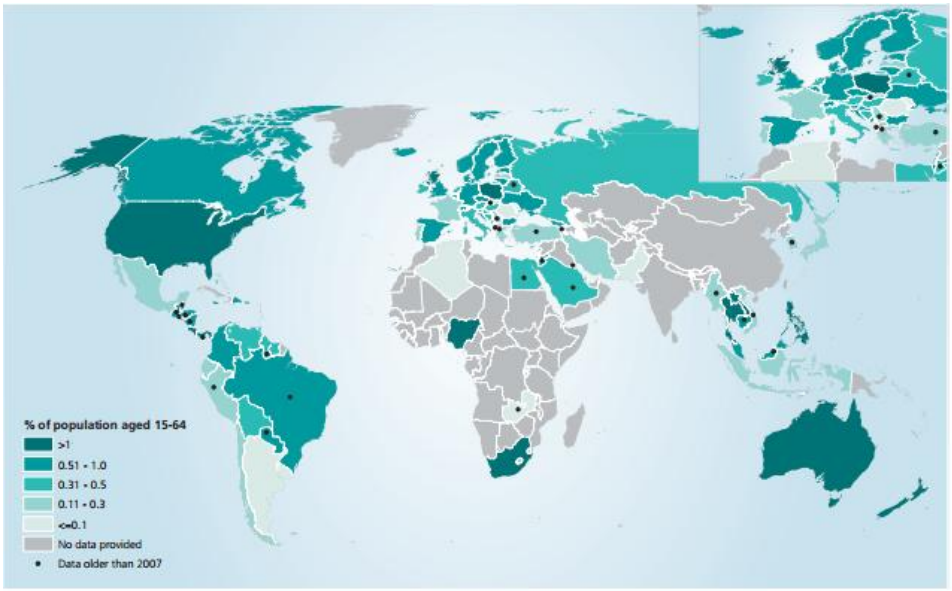

Figure 6 - Use of ATS in 2011. Information taken from World Drug Report, 2013.

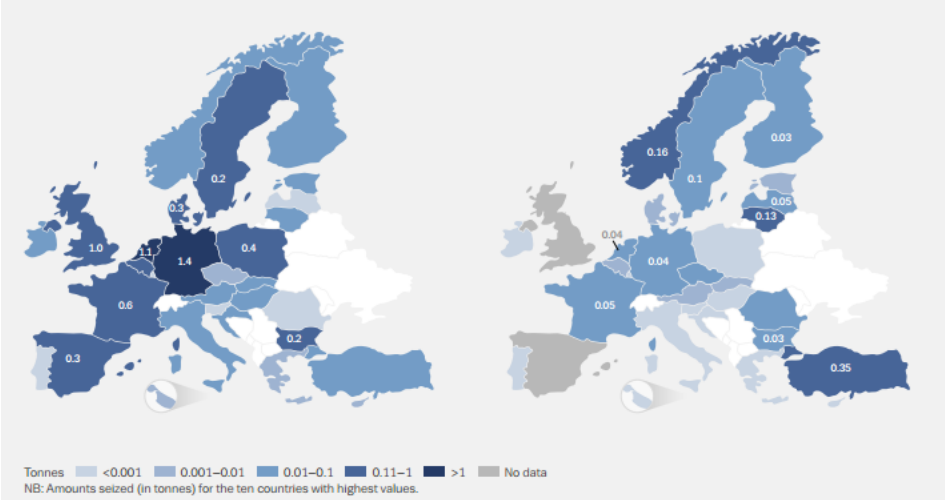

Figure 7 - Quantity of amphetamine (left) and methamphetamine (right) **seized**, 2011 Figure taken from World Drug Report 2013.

2.3 Methamphetamine: pharmacokinetics and pharmacodynamics

Amphetamine was first synthesized in 1887 in Germany, at the Berlin University. However, it was not used clinically until the 1920's, after the discovery of a more potent, easy to make and more lipophilic derivative Methamphetamine (*N*-methyl-*O*-phenyllisopropylamine) in Japan.

During the World War II, amphetamines were widely used for diverse clinical conditions. In the 1950's, METH was prescribed to induce weight loss and to manage depression, but it rapidly became a drug of abuse and was made illegal for most uses in 1970 by the US government (Halpin, Collins, & Yamamoto, 2014). Nowadays, METH is prescribed to treat children with ADHD and severe obesity. The therapeutic dose used for ADHD children is 5-30 mg/day, whereas METH abusers need 40-60mg/day to achieve the euphoric state (Kish, 2008).

A different way to obtain METH is through the reduction of pseudoephedrine or the condensation of phenylacetone and methylamine (Cho & Melega, 2002; Cho, 2012), producing a lipid-soluble pure base form. For being extreme volatile, this product is often converted to a water-soluble methamphetamine-HCl powder, dealt in the streets by the name of 'speed', 'crank' or 'crystal' (Derlet, Heischober, & Linda, 1988).

Acute METH administration depresses appetite, increases wakefulness, alertness and energy levels; it causes euphoria, heightened libido and feelings of self -esteem and –confidence (Meredith et al., 2004). Methamphetamine also activates the cardiovascular system (increased heart rate and blood pressure) and, for this reason, can cause death at high doses. Chronic use of METH contributes to anxiety, depression, aggressiveness, social isolation, psychosis, mood disturbances and psychomotor dysfunction. Neuropsychological studies of chronic METH users detected deficits in attention span, working memory, and decision-making. These neuropsychiatric complications are

related to drug-induced neurotoxic effects including damage to dopaminergic and serotonergic terminals.

2.4 Routes of administration

The intensity and duration of the psychotropic effects depend on how fast the drug arrives to the CNS. Abusers use different administration methods and ways, in order to enhance the drug bioavailability and achieve rapid and intense "high" (McAvoy, 2009). METH can be taken by mouth (oral), injection (intravenous), smoking/inhalation ('chasing the dragon'), snorting (intranasal) or rectally ('shelving'). Smoked methamphetamine is rapidly absorbed thought the lungs and reaches the brain in 6-8 seconds. Intravenous administration produces peak brain uptake in four to seven minutes. These drug forms have the highest bioavailability, allowing elevated drug concentration at the CNS, and, thereby, a very rapid and intense "high" that lasts a couple minutes. For this reason, smokable and injectable METH forms are highly addictive and have enhanced overdose risks.

Oral and intranasal methamphetamine have a slower absorption and onset of effect (30-45 minutes), have a longer peak effect (about 3 hours post dose), and a more gradual decline from peak. The peak intensity of effect is weaker than with smoked or intravenous administration because less active drug reaches its site of action in the brain. The elimination half-life of METH ranges from 10 to 12h (Schepers et al., 2003).

2.5 Mechanism of action

In the brain, a primary action of METH is to elevate the extracellular levels of monoamine neurotransmitters (dopamine, serotonin and noradrenaline) by promoting their release from nerve endings (Kish, 2008). Due to its lipophilicity METH permeates monoamine terminals triggering monoamine leakage from the vesicles, thus increasing their intracellular concentration. This excess of neurotransmitters is then carried by transporter molecules out of the terminals into the synapse. For example,, high extracellular concentration of dopamine in the mesolimbic pathway causes feelings of pleasure and euphoria (Hsieh et al., 2010).

METH is highly addictive because it works directly on the brain's reward pathway, making the user feel intense pleasure and exhilaration.

2.6 Methamphetamine neurotoxicity: shedding light into brain glucose handling

There is a mounting body of evidence of preclinical and clinical data suggesting that perturbed glucose handling is a hallmark of METH-induced neurotoxicty (Kobeissy et al., 2012).

a) Preclinical evidence

In order to study the toxic effects of METH administration, numerous animal models have been developed over the years. These methods range from single exposure acute models to extended chronic models.

Among the well-characterized models is the acute model regimen. Rodents are exposed to a large dose of METH within a single-day period which can be achieved by administering a single large bolus of METH or through repeated doses of METH during a single day. Administering repeated doses allows for the assessment of very high doses of METH without the potential of premature animal death due to sudden overdose of METH.

The chronic model or the escalating dose model starts with low nontoxic doses and then increases METH concentration administered over the time period until larger neurotoxic doses of METH are achieved. This increase can either be consistent across days, which simulates the progression of use observed in humans, or end with a large challenge dose on the final day, which is often used to assess any protective effects of previous exposure to METH.

Huang et al. (1999) used the 2-[^{14}C]deoxyglucose (2DG) method to analyze the effects of repeated METH administration (12.5 mg/kg, i.p., 4 times every 2 hr within a day) 14 days and 60 days after drug administration on rat brain glucose utilization. The

results showed a widespread decrease in the regional cerebral glucose utilization at both time-points. In a different study, a daily dose of 15mg/kg for 5-6weeks almost completely inhibited glucose transport into the frontal and occipital cortex of mice, along with diminished levels of $GluT_1$ expression (Muneer et al., 2011).

The effects of acute intravenous administration of methamphetamine (0.5-2.5mg/kg) on rates of local cerebral glucose utilization in freely-moving rats were also analyzed using the 2-DG method. Methamphetamine administration resulted in widespread dose-dependent increases in glucose utilization within structures of the extrapyramidal motor system including globus pallidus and substantia nigra reticulate. These effects were correlated with the increase in locomotor activity that was seen simultaneously in the same animals (Pontieri, Crane, Seiden, Kleven, & Porrino, 1990).

A single administration of methamphetamine to Wistar rats induced dopaminergic nerve activation, ATP consumption and an increase in mitochondrial respiratory chain function in both the striatum and cortex (Shiba et al., 2011). This is consistent with a previous study demonstrating that high METH doses significant and acutely increased extracellular concentrations of lactate in striatum and prefrontal cortex (Stephans et al., 1998).. There are also in vitro studies tackling this metabolic issue. In fact, cells from elective abortus specimens of human fetal brain tissues were exposed to METH, showing biphasic effects on astrocytic glucose uptake: while 20 mM METH increased the glucose uptake, 200 mM inhibited it (Muneer et al., 2011a). Another study by these authors looked at the dose-dependent effect of METH (5 - 500 μM) exposure for 24 h on glucose uptake by primary human brain endothelial cell (hBEC) culture (Muneer et al., 2011b). These authors provide data indicating that there was an insignificant increase in glucose uptake by hBECs following exposure to 5-20 μM of METH. However, the higher concentrations 50-500 μM of METH decreased

dose-dependently the glucose uptake by hBECs. Also, a time-dependent study showed that 20 μM METH gradually decreased the glucose uptake by hBECs with exposure time, suggesting that low concentration of METH may activate $GluT_1$ in acute condition but in long-term it impairs $GluT_1$.

a) Clinical evidence

Several clinical studies have noted cerebral glucose hypometabolism in human METH abusers.

For example, relative regional cerebral glucose metabolism (rCMRglc) changes in abstinent methamphetamine users were explored by Kim et al. (2005). These authors showed that METH users had lower rCMRglc levels in the right superior frontal white matter, relative to healthy comparison subjects. Age or smoking habits did not interfere with the metabolism of glucose.

Also, the 2-[^{14}C]deoxyglucose method to analyze the effects of repeated METH administration (12.5 mg/kg, i.p., 4 times every 2 hr within a day) 14 days and 60 days after drug administration. Chronic treatment reduced subcortical glucose metabolism in rats. The regions with decrease metabolism included all the extrapyramidal systems, the hippocampus formation and dorsal raphe nucleus (Huang et al., 1999).

3. Major Depressive Disorder

Major depressive disorder (MDD) is also known as major depression or unipolar depression. The term unipolar refers to the presence of one pole, or one extreme of mood - depressed mood. This may be compared with bipolar disorder which has the two poles of depressed mood and mania (i.e., euphoria, heightened emotion and activity) (reference).

According to the National Institute of Mental Health, MDD currently affects about 6.7% of the U.S. population over age 18. In adults, major depressive disorder affects twice as many women as men. For both genders it is most common in those who are 25-44 years of age, and least common for those over the age of 65 (Kessler et al., 2005). In children, clinical depression affects girls and boys at about the same rate. Within an entire lifetime, major depression will affect 10-25% of women and 5-12% of men. The average age for developing the illness seems to be in a person's mid-20's, but MDD can affect teens, children and older adults, though it frequently goes undiagnosed and untreated in these populations. Those with a parent or sibling who has had major depression may be 1.5 to 3 times more likely to develop the condition than those who do not. Studies of clinical samples suggest that 10–30% of individuals with MDD develop a chronic course despite adequate treatment, indicating that chronic major depression is a major public health problem, and is expected to be the second greatest cause of disability by 2020 (Blanco, 2010). Decreased energy and focus, sleep deprivation, headaches and body aches, the feelings of sadness, hopelessness, loss of interest or pleasure (anhedonia), guilt, shame and anger, appetite changes and thoughts of suicide are among the most common depressive symptoms (APA, DSM-IV-TR, 2000).

Only 50-70% of MDD cases report complete lack of symptoms and unpleasant secondary effects, such as nausea, insomnia, decreased sex drive, weight gain, diarrhea and constipation with long-term treatment using antidepressants (Berton & Nestler, 2006).

3.1 Depression: focusing on brain glucose metabolism disruption

Although perturbations in serotonergic system play an important role in the pathophysiology of major depression (Cowen, 2008), the link between brain metabolism alterations and depression is also under close scrutiny (Videbech, 2000).

Functional neuroimaging techniques, including fluorine-18-fluorodeoxyglucose positron emission tomography (18F-FDG PET) have helped to delineate regional differences in metabolic activity between depressed and non-depressed subjects. Studies have shown abnormalities of regional cerebral blood flow (CBF) and glucose metabolism in multiple prefrontal cortical and limbic structures that have been more generally implicated in emotional processing (Drevets et al., 2002; Kennedy et al., 2007) . In fact, these authors showed that the mean metabolism was increased in the left and right lateral orbital cortex/ventrolateral prefrontal cortex (PFC), left amygdala, and posterior cingulate cortex, and decreased in the subgenual ACC and dorsal medial/dorsal anterolateral PFC in the unmedicated depressives relative to controls. Following treatment, metabolism significantly decreased in the left amygdala and changes in the orbital and posterior cingulate cortices approached significance.

Kimbrel et al. (2002) reported a decreased cerebral glucose metabolism (rCMRglu) in right prefrontal cortex and paralimbic/amygdala regions as well as bilaterally in the insula and temporoparietal cortex from depressed patients when

compared with healthy control subjects using PET imaging . Decreased glucose metabolism was also observed in the parietal cortex of depressed patients using 18F-FDG PET (Bive et al., 1994; Hosokawa, Momose, & Kasai, 2009).

3.2 Depressive behavior during early methamphetamine withdrawal

It is widely known that substance abuse and mood disorders are intimately associated. Notably, the initial phase of withdrawal in chronic METH users, known as *crash*, is characterized by psychiatric and somatic symptoms (Cryan et al., 2003). In fact, during withdrawal, chronic METH abusers have more psychiatric complaints than physical manifestations, namely anxiety, depression with severe dysphoria, anhedonia, irritability and melancholia, social isolation, fatigue with hypersomnia, psychomotor dysfunction, mood disturbances, impaired social functioning, intense craving for the drug and even paranoia or aggression, as well as attention deficits and memory in making decisions (Scott et al., 2007; Darke et al., 2008). While the abstinence syndrome seems to be transient, symptoms associated with MDD can persist for months (Barr et al., 2006).

Moreover, when abstinent for 1 week, METH abusers showed more severe self-reports of depressive symptoms than control subjects, and these self-reports covary with relative uptake of the radiotracer 18F-FDG in anterior cingulate cortex and amygdala evaluated by PET (Berman et al., 2008).

However, the temporal relationship between depression and METH use is unclear: it is unknown whether experiencing depressive symptoms promotes METH use, whether depression results from or is enhanced by METH use, or whether it is bidirectional (Sutcliffe et al., 2009).

This relationship between depressive disorders and METH has been also studied in animal models. For example, a depressive-like behavior was reported in mice during an early METH withdrawal (4-7 days), after short (1h) and extended (6h) access to methamphetamine self-administration (0.05 mg/kg) (Jang et al. 2013).

Also, Kitanaka et al. (2012) suggested that a single administration of methamphetamine to mice early in the light period decreases running wheel activity The same group reported increased anxiety during mice withdrawal, after ten days of consecutive METH administration (1.0 or 2.5 mg/kg; twice a day) (Kitanaka et al., 2010). A transitory reduced locomotive activity was also reported in mice, 3 days after METH subcutaneous injection (10mg/kg; 4 injections every 2h). However, 1 week after the drug administration animals displayed normal locomotor activity.

A depressive-like behavior was also observed by Cryan et al. (2003), 24h after AMPH administration (5mg/kg/day, 7days) due to increased immobility of mice on the tail suspension test.

Finally, our group recently demonstrated that a single high neurotoxic dose of METH (30 mg/kg i.p.) evoked a long-lasting depressive-like behavior as gauged by increased immobility in the tail-suspension task measured within the first week (3 days) and 7 weeks post-METH treatment (Silva et al., 2014). This long-lasting depressive-like profile was accompanied by monoaminergic (dopamine and serotonin) disruption in both striatum and frontal cortex.

Importantly, psychostimulant withdrawal in rodents seems to provide the basis for the development of an animal model of depressive symptoms, such as despair, anhedonia and lethargy (Paulson, Camp, & Robinson, 1991; Cryan et al., 2003).This would allow the screening of new pharmacological approaches on the search for a efficient treatment to reverse the abstinence syndrome (Barr, Markou, & Phillips, 2002).

Chapter 2

Aim

Aim

The aim of this work is to further characterize the negative emotional states of mice and the underlying frontal cortical and hippocampal metabolomic profile probed 3 days following a single high neurotoxic METH dose (30 mg/kg) – early withdrawal.

Chapter 3

Materials and Methods

1. Animals

Ten-week old male C57BL/6 mice weighing 21–26g were obtained from Charles River Laboratories (Barcelona, Spain) (Figure 8) and maintained in the animal house of the Faculty of Medicine, University of Coimbra (FMUC) with a standard 12:12-hour light-dark cycle and controlled humidity, temperature (22 ± 1° C), and pathogen-free conditions. Animals were grouped in 4 cages (4 per cage) with food and water provided *ad libitum*. Their weight was monitored before the administration of METH and before the sacrifice, in order to evaluate the anorexic effect of the drug.

All experiments were approved by the Institutional Animal Care and Use Committee from Faculty of Medicine, Coimbra University, and were performed following the European Community directive (2010/63/EU). The animal procedures were performed in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press 1996).

All efforts to minimize animal suffering and to use the smallest possible number of animals were made.

Figure 8 - Example of C57BL/6 mouse. Image from the Jackson Laboratory.

2. Drugs and Chemicals

We were issued permission by INFARMED, Portugal (National Authority of Medicines and Healths Products) to import methamphetamine.HCL (METH) from Sigma-Aldrich (St. Louis, MO, USA). The other used chemicals (ultrapure and pro analysis quality) were purchased from Santa Cruz Biotechnologies, Inc., Merk KGaA (Darmstadt, Germany), Merck Millipore, Merk KGaA, Sigma-Aldrich and Abcam (Cambridge, UK).

3. Experimental Design

A total of 16 animals were randomly divided into 4 cages, with 4 animals each: 2 cages having control groups (SAL) and other 2 cages having methamphetamine groups (METH).The animals were previously identified with ink stripes on the tail and housed in their respective cages, and were allowed one day of adaptation to the animal house before the experimental procedure.. Mice weight was recorded before the administration of the neurotoxin and 72h post-injection, using an analytical balance (Kern CB 6 K1, Germany). Control groups (SAL) were administered a saline solution (NaCl 0.9%, 250µl.), while METH groups were injected with 30 mg/kg methamphetamine.HCL (METH; 3mg/ml). Animals were injected with a single intraperitoneal dose (i.p.). Immediately following METH injection, animals were extremely agitated, sweated and presented bristly tail and coat and repetitive rapid movements. This is consistent with what is reported in the literature (Meredith et al. 2005). 3 days following METH administration, behavior tests were performed and animals were, finally, sacrificed for dissection of discrete brain regions (namely frontal cortex and hippocampus).

4. Behavioral Tests

Behavioral tests that included elevated-Plus Maze (EPM) Splash and Forced-swim test (FST) and were performed72 hours after administration of METH (30 mg/kg, i.p.) or saline to assess mice negative emotional states. The tests were performed in the listed order to prevent carryover effects. Tests were performed between 9 am and 6pm, in a sound-attenuated room lit with low intensity Light (12 lx). Animals were transferred to this room 1h before the start of the testing so that animals can get used to the environment. The behavior was monitored by a video camera positioned above the equipment and the images were subsequently analyzed with the system of video monitoring ANY Maze (Stoelting Co., Wood Dale, IL, USA) by an experienced experimenter who was unaware of the experimental group being tested.

4.1 Elevated-Plus Maze

The Elevated-Plus Maze is often used to evaluate the anxiolytic or anxiogenic drug effects in mice (Lister, 1990).

This test was performed in a black acrylic apparatus (LE 848 PANLAB, Barcelona, Spain) placed 55 cm above the floor. The four arms heighted 18 cm long and 6cm width. Two opposite arms were surrounded by gray opaque walls, 15cm high (closed arms), while the other two had no walls around it (open arms) (Figure 9). Each animal was placed in the center of the apparatus, facing an open arm, and observed during 5 minutes, by an experienced person. The following parameters were evaluated: time spent and the number of entries into open and closed arms. An animal was considered to entry an arm whenever all four limbs were inside an arm. An anxiolytic-like behavior was defined by a decreased proportion between the number of entries on the open arms divided by the total number of entries in the four arms and also by a decrease in the time spent on the open arms, compared to the total time spent in both type of arms. The total number of entries in the closed arm was used as a measure of locomotor activity.

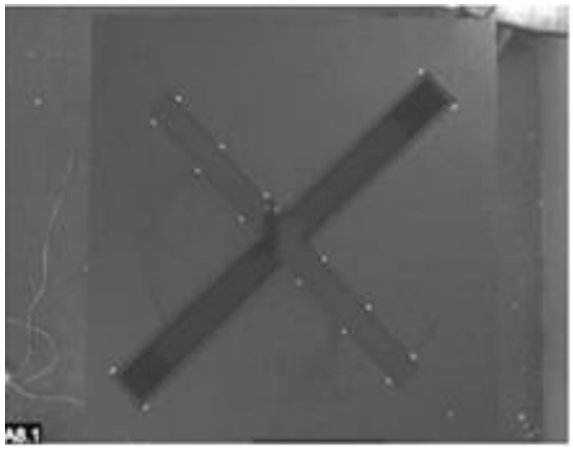

Figure 9 - Elevated-Plus Maze: Mice are placed in the centre, facing an open arm.

4.2 Splash

This test protocol was used to evaluate the impact of methamphetamine administration on grooming behavior in mice as an index of self-care and motivational behavior phenotype of the experimental groups and was adapted from Yalcin et al. (2005). For this purpose, 10% sucrose solution was squirted on the dorsal coat of mice in their home cage. Following this viscous solution dirtying the mice fur, animals initiate grooming behavior. The mice grooming time was recorded in an acrylic chamber (40 x 40x 40 cm) during 6 minutes (Figure 10).

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

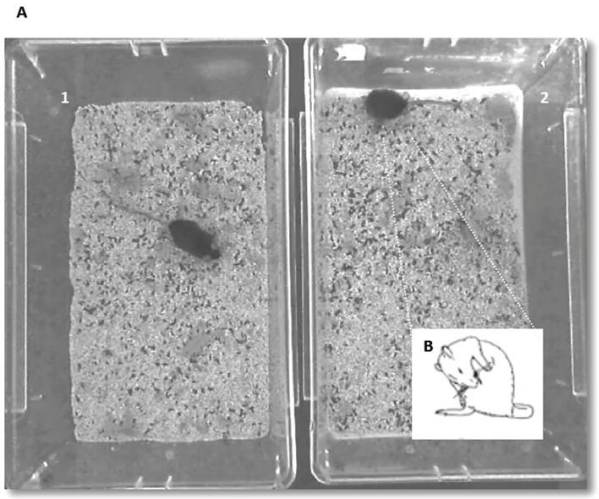

Figure 10 - Splash Test: A - Mouse in cage 2 is grooming his back fur (sucrose grooming); B - schematic representation of sucrose grooming.

4.3 Forced-swim test

The Forced-swim test has been used as a model predictive of antidepressant effect (Cryan et al., 2003). The test procedure was carried out according to Porsolt et al. (1977) with some modifications. Mice were individually forced to swim in open cylindrical container (21 cm height × 12 cm internal diameter) containing fresh water till a height of 15 cm at 23±1 °C; the total duration of immobility was recorded during a 6 minute period (Figure 11). The water was changed and the cylinder refilled with clean water after each mice.

During the 6 min swimming test session, a trained observer recorded the total immobility time (i.e. the time spent floating in the water without struggling, making only those movements necessary to keep its head above water level) Since animals tend to immediately struggle when dropped in the cylinder, the same trained experimenter also recorded the previous behavioral responses during the last 4 minutes of the test, in order to discard the initial agitation caused by the sudden drop in water. An increased duration of immobility time is indicative of a depressive-like behavior (Porsolt, 1977).

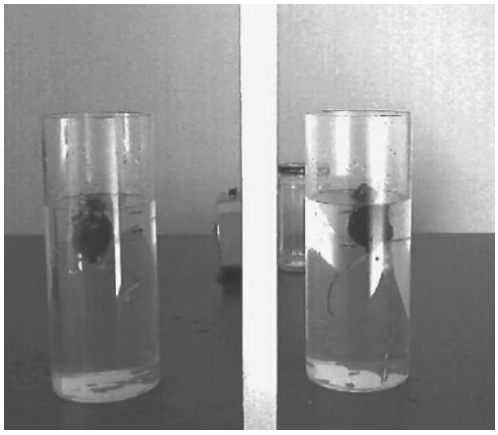

Figure 11 - Forced Swim Test: Mice placed into an open cylindrical container.

5. Frontal cortex and Hippocampus isolation

Following behavioral tests, animals were sacrificed by cervical dislocation and decapitated. The brains were rapidly removed and immediately frozen in liquid nitrogen and stored at -80 ° C later utilization. The hippocampus and the frontal cortex were later dissected on ice based on the coordinates for the mouse brain described by Paxinos & Franklin (2004). Biological samples from the right hemisphere were used for ^{1}H-NMR analyses while the ones from the left hemisphere were used for Western Blotting analyses.

6. Proton HRMAS

Proton (^{1}H) high rotation magic angle spinning (HRMAS) spectroscopy was performed in the different brain regions using a 800 MHz Brucker NMR spectrometer equipped with a 4 mm HR MAS Triple H/C/N probe-head, specific for high resolution liquid or semi-solid samples. A 1D Carr–Purcell–Meiboom–Gill (CPMG) NMR sequence was used. Typical acquisition parameters included a 5.45 seconds acquisition time, defining a 12 kHz sweep width, and a recycle delay of 2 seconds. A total of 256 scans were averaged to allow good signal to noise for metabolite quantification. Before Fourier transformation each FID was multiplied by 0 0.5 Hz Lorentzian to improve signal to noise. Spectral integration for metabolite quantification was performed using NUTSpro^{TM}.

7. Western Blotting

The frontal cortices from left hemispheres were homogenised in 400 µL of RIPA buffer (NaCl 150 mM; Tris-HCl 50 mM, pH=8.0; EGTA 5 mM; Triton X-100 1 %; sodium deoxycholate (DOC) 0.5%; sodium dodecyl sulfate (SDS) 0.1 %) with protease and phosphatase inhibitors cocktail (PMSF 1 mM, DTT 1 mM, chymostatin 1µg/mL, leupeptin 1 µg/mL, antiparine 1 µg/mL, pepstatin A 5 µg/mL, sodium fluoride 50 mM e sodium orthovanadate 1 mM (Sigma-Aldrich, Sintra, Portugal)). This cocktail was added to the lyses buffer immediately before its use.

Brain tissue was homogenized by ultrasounds (3 pulses of 10s), diving the samples in ice between pulses, in order to avoid the biological samples to overheat. Protein lisates were then centrifuged at 13000 rpm (15493 x g), for 15 minutes, at 4ºC. Supernatants (total extracts) were collected and stored at - 80°C. Protein quantification was determined according to the Bicinchoninic acid assay (BCA - Thermoscientific®). Samples were denatured at 37ºC for 60 minutes ($GluT_1$) or at 98 ºC for 5 minutes (Synaptophysin and Syntaxin 1) in denaturing solution 6x diluted (Tris-HCl, 0,5M, pH 6,8; SDS 10% (m/v); glycerol 30% (v/v), DTT 0,6M, bromophenol blue 0,01% (m/v)). Equal amounts of protein were loaded into the gels and separated by electrophoresis on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% gels. Then, proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Millipore, Madrid, Spain), and blocked with 5% non fat dry milk in phosphate-buffered saline solution (PBS, in mM: 137 NaCl, 2.7 KCl, 4.3 Na2HPO4, 1.47 KH2PO4; pH 7.4) containing 0,1% Tween-20 (PBS-T) for 2 h at RT.

The membranes were probed with primary antibodies (Table 1) overnight at 4 °C. Membranes were then incubated with alkaline phosphatase-conjugated IgG secondary (Table 1) prepared in PBS-T for 1h at RT. Finally, membranes were

visualized on an imaging system (Thyphoon FLA 9000, GE Healthcare) using an enhanced chemifluorescence detection reagent (ECF, GE Healthcare). To confirm equal protein loading and sample transfer, membranes were reprobed with mouse anti-β-actin 1:5000 (Sigma-Aldrich) or mouse anti-tubulin 1:10000 (Sigma-Aldrich). Densitometric analyses were performed using the Image Quant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA) and results were expressed as percentage of control and presented as mean ± standard error (SEM).

Table I - Primary and secondary antibodies used for Western Blot analysis.

Antibody	Molecular Weight (kDa)	Quantity of Protein (µg)	Dilution (µL)	Reference
Mouse anti-Syntaxin 1	37	20	1:5000	SynapticSystems (110 011)
Mouse anti-Synaptophisin	38	10	1:1000	Sigma-Aldrich (S5768)
Rabbit anti-$GluT_1$	54	20	1:500	Millipore (07-1401)
Mouse anti-Tubulin	52	-	1:10000	Sigma-Aldrich (T6199)
Mouse anti-β-actin	42	-	1:5000	Sigma-Aldrich (A5316)
Goat anti-mouse	-	-	1:10000	Sigma-Aldrich (A3582)
Goat anti-rabbit	-	-	1:1000	GE Healthcare (NIF1317)

8. Statistical analyses

The results are expressed as mean ± SEM. Spleen and supra-renal glands weight, behavioral and neurochemical data were analyzed by t-test, where * $p<0,05$; ** $p<0,01$ e *** $p<0,001$.

The statistical analyses were performed using GraphPad Prism 6.0 software.

Chapter 4

Results

1. Mice emotionality at early METH-withdrawal

1.1 Anxiety-like behavior of methamphetamine-injected mice probe by elevated-plus maze

No anxiolytic-like behaviour was associated with the METH injection, because time spent (Figure 12 (A)) and number of entries into open arms (Figure 12(B)), by the METH group was not significantly different from the control group ($p>0.05$). Moreover, no significant differences among groups was observed in number of entries in the closed arms suggesting that the METH group did not show locomotor impairment (Figure 12 (C); $p>0.05$).

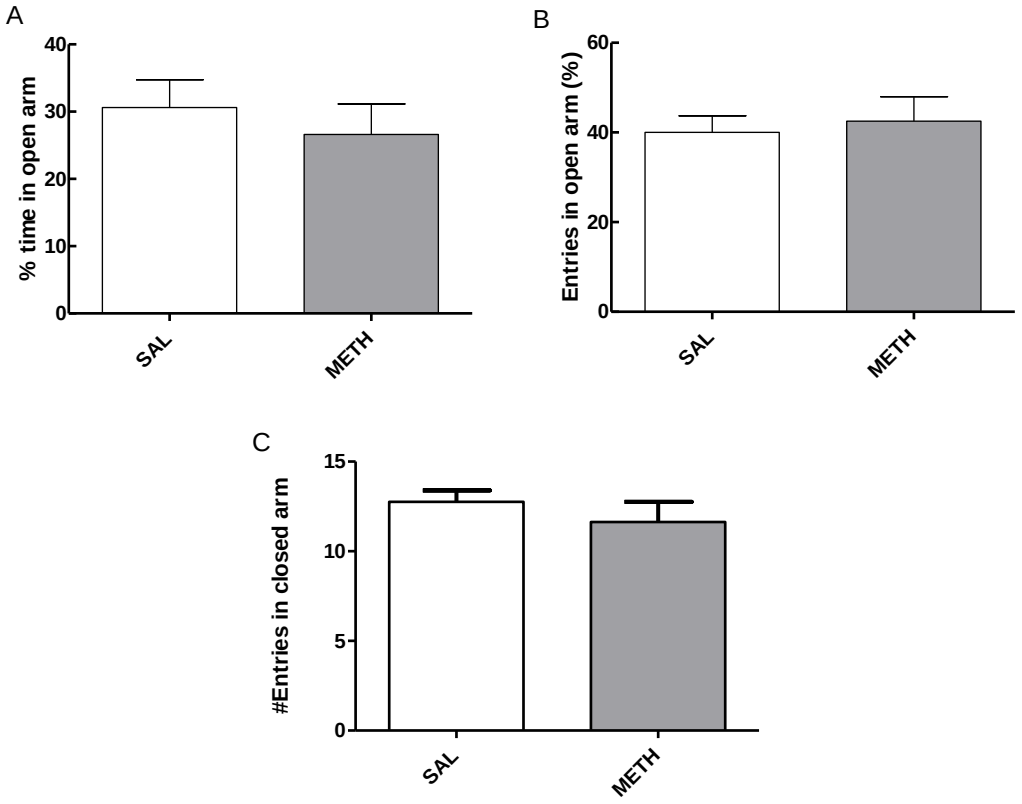

Figure 12 - Anxiety-like behaviour behaviour in mice injected with a single dose of METH (30mg/kg, i.p.) or saline (SAL) evaluated by elevated plus maze test. The figure shows the percentage of time spent on open arm (A), the percentage of entries into open arm (B) and number of entries in closed arms (C) by mice 72h post-injection. These behavioural parameters were recorded for 5 min. The results are expressed as mean ± S.E.M. of 8 animals per group.

1.2 Depressive-like behaviour of methamphetamine-injected mice

a) Splash test

Mice anhedonic-like phenotype was assessed using the Splash test that allowed the measurement of grooming behavior for 5 minutes, 3 days post-injection. METH-injected animals displayed a significantly decreased total grooming time when compared to control animals (Figure 13: $p<0.05$).

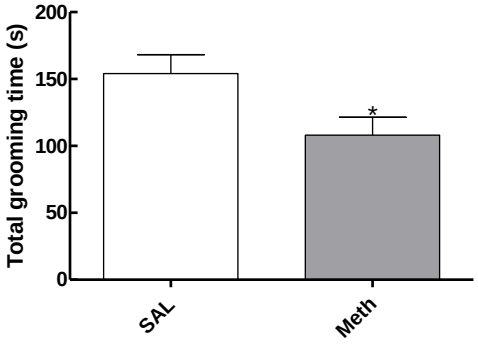

Figure 13 - Anhedonic-like behaviour in mice injected with a single dose of METH (30mg/kg, i.p.) or saline (SAL) evaluated by the splash test. The figure shows the total grooming time of mice 72 h post-injection. These behavioural parameters were recorded for 5 min. The results are expressed as mean ± S.E.M. of 7-8 animals per group. *$p<0.05$ versus saline group using unpaired t-test.

b) Forced-swim test

Mice despair-like behaviour was probed using forced swim-test for 6 minutes, 3 days post-METH injection. METH-injected mice did not show a despair-like behavior as gauged by total immobility time as well as immobility time measured during the last 4 minutes not being statistically different from those shown by their controls (Fig.14 A, B; $p>0.05$).

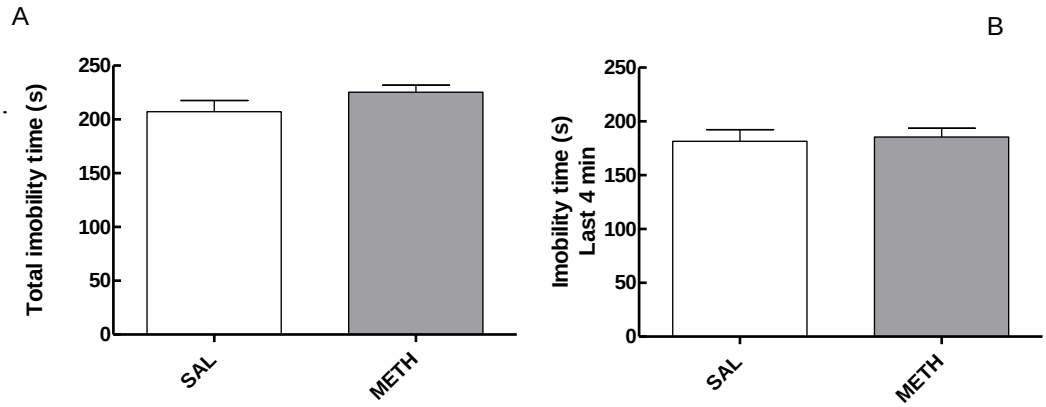

Figure 14 - Despair-like behaviour in mice injected with a single dose of METH (30mg/kg, i.p.) or saline (SAL) evaluated by the forced swim test. The figure shows the total immobility time (6 minutes) (A) and the immobility time during the last 4 minutes (B) of mice 72 h post-injection. The results are expressed as mean ± S.E.M. of 8 animals per group.

2. Effect of Methamphetamine on body and spleen weight

The administration of a single neurotoxic dose of METH did not alter the animals' body weight, 72h post- injection (Figure 15 A).

Mean spleen masses were not significantly different between groups (66,4 ±3,0 and 72,7 ± 4,9 mg for control and METH, respectively; $p>0.05$). This difference remained not significantly different, even when corrected for individual body weight. (Figure 15(B,C), $p>0.05$).

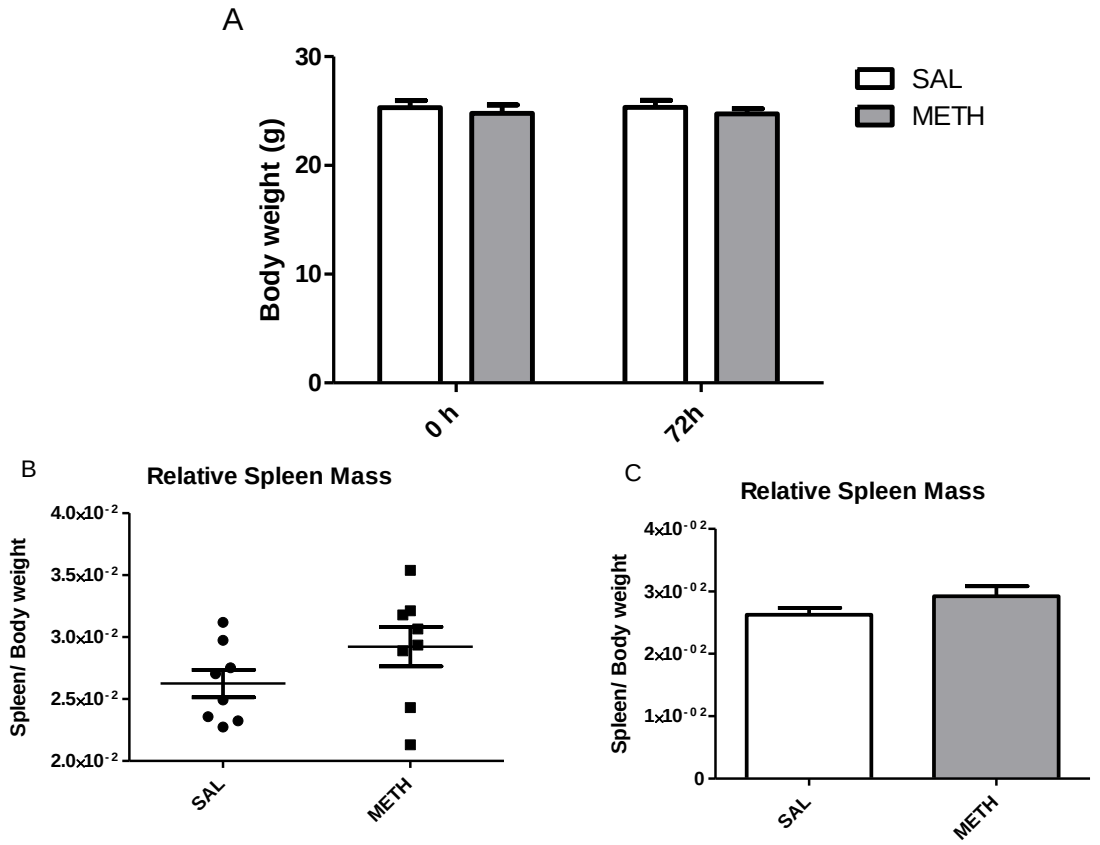

Figure 15 - Body and spleen weight from mice injected with a single dose of METH (30mg/kg, i.p.) or saline (SAL). The figure shows mice body weight 0 and 72h post-METH (A) and relative spleen mass (spleen-to-body weight ratio) (B and C) 72h post-METH. Panel (B) represents scatter plot to highlight data dispersion regarding relative spleen mass. The results are expressed as mean ± S.E.M. of 8 animals per group.

3. Metabolic profiling of frontal cortex and hippocampus from METH-injected mice: metabolomics approach

Figures 16 and 17 show representative ^{1}H-NMR spectra obtained from frontal cortices and hippocampi of mice 3 days post-METH or SAL injection. METH-treated mice showed a decrease in both n-acetylaspartate (NAA)/Cre and lactate/alanine ratios (Figure 18(i,ii); $p<0.01$) on frontal cortex when compared to control mice, whereas no differences were seen on the hippocampus between groups regarding these metabolites (Figure 19 (i,ii); $p>0.05$), All the other analyzed metabolites were not significantly different between groups in both studied brain regions (Figs 18 (iii-viii), 19 (iii-viii); $p>0.05$).

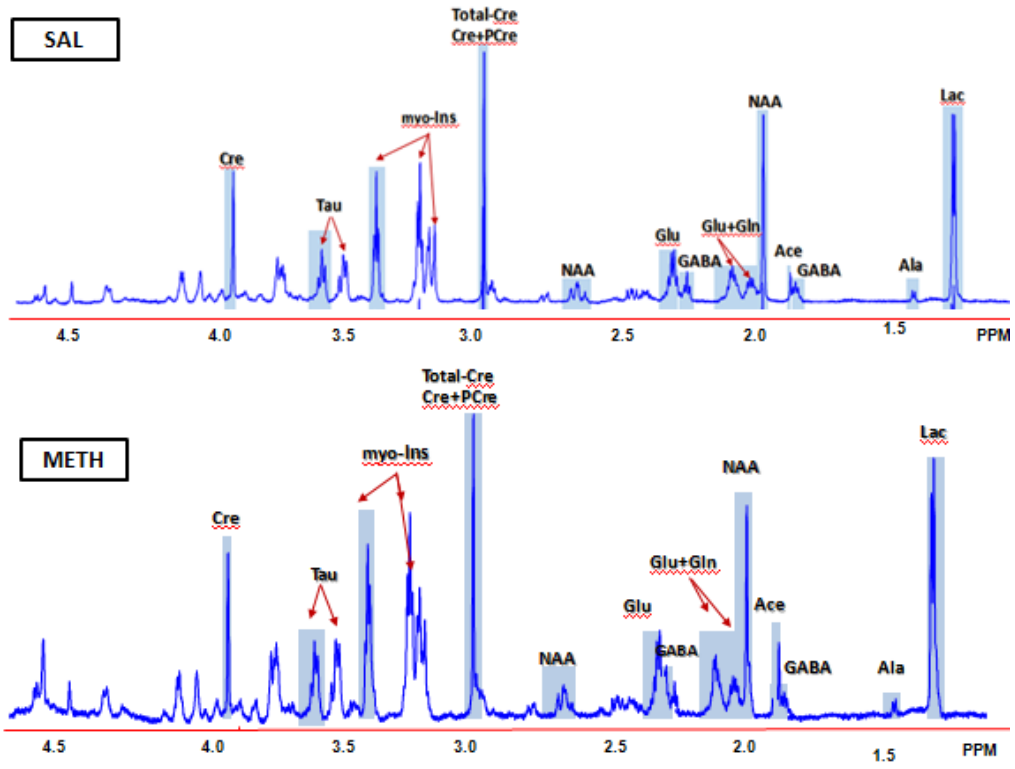

Figure 16 - A typical 800 MHz CPMG 1H NMR spectra of frontal cortex from mice exposed to METH/saline.

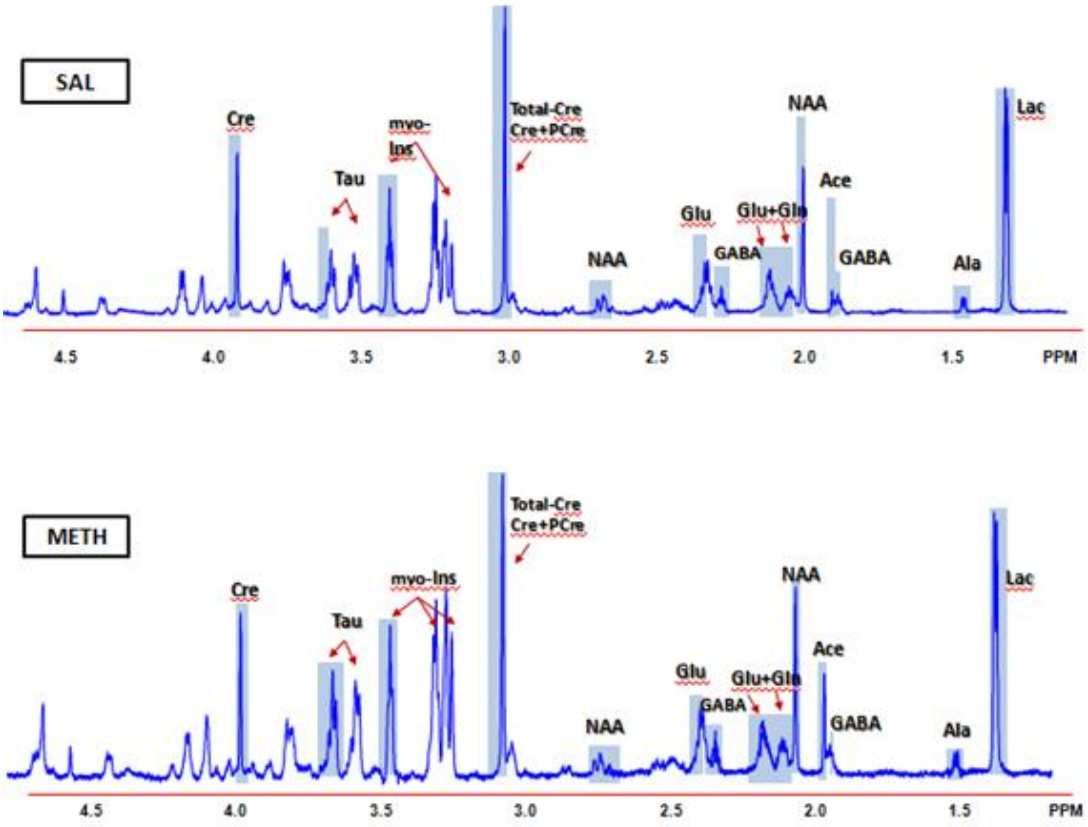

Figure 17- A typical 800 MHz CPMG ^{1}H NMR spectra of hippocampus from mice exposed to METH/saline.

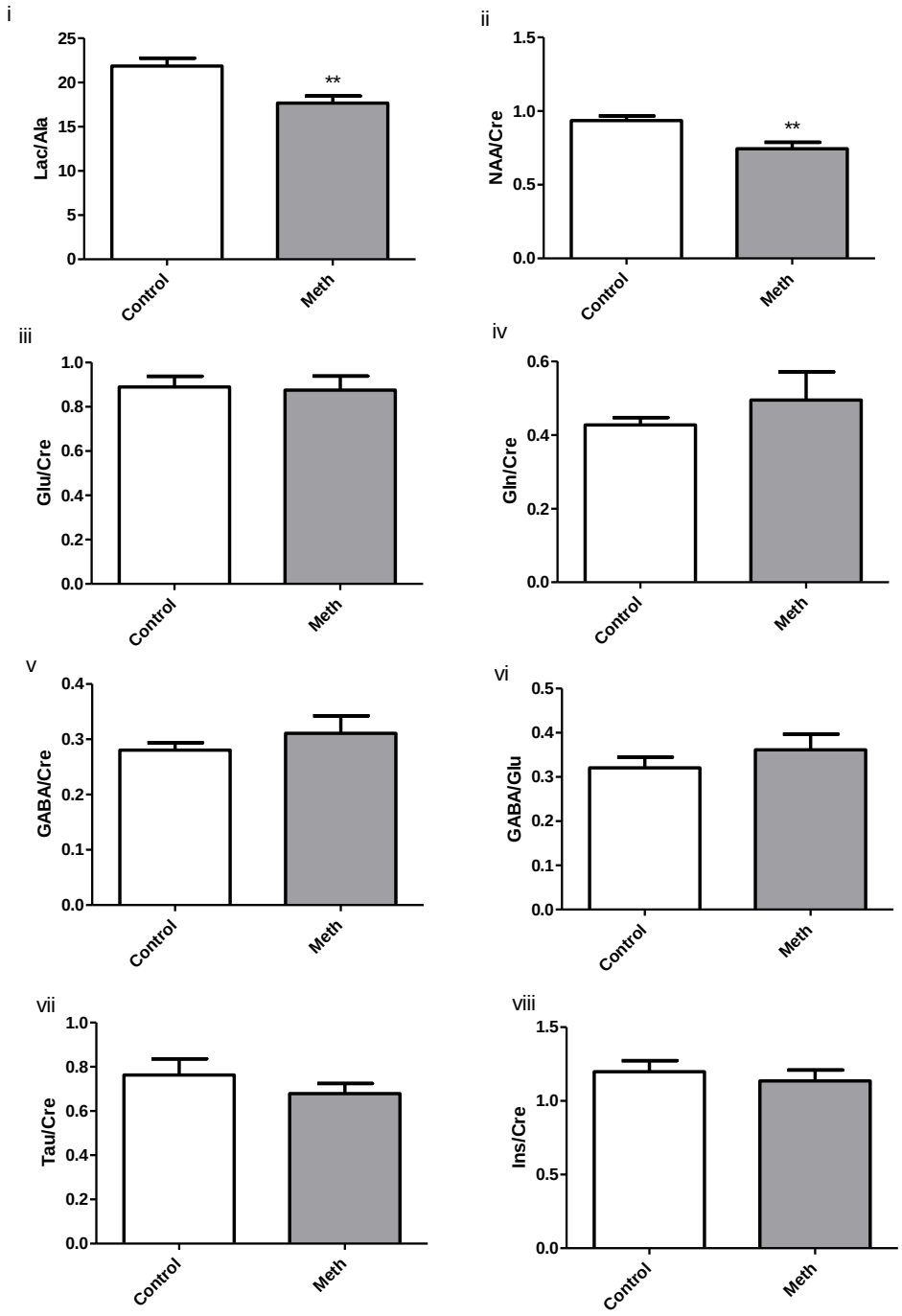

Figure 18 - ^{1}H NMR spectral integration for frontal cortex metabolite quantification from mice injected with single dose of METH (30mg/kg, i.p.) or saline (SAL). Figure shows (i) n-acetyl-aspartate, (ii) lactate/alanine ratio, (iii) glutamate, (iv) glutamine, (v) GABA, (vi) GABA-glutamate ratio, (vii) taurine and (viii) inositol, The results are expressed as mean ± S.E.M. of 6-8 animals per group. **p<0.01 versus saline group using unpaired t-test.

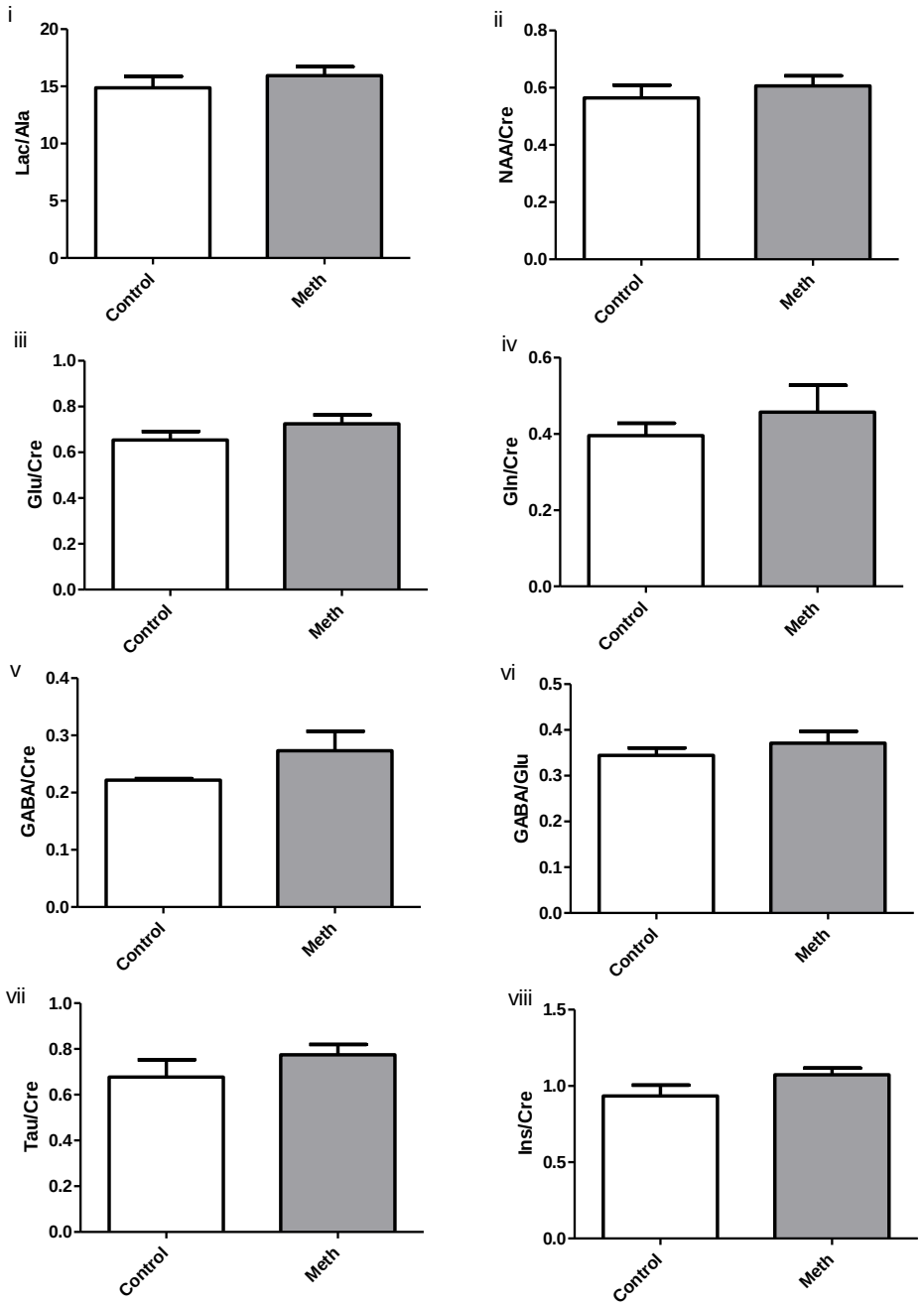

Figure 19 - ^{1}H NMR spectral integration for hippocampal metabolite quantification from mice injected with single dose of METH (30mg/kg, i.p.) or saline (SAL). Figure shows (i) lactate/alanine ratio, (ii) n-acetyl-aspartate/creatine ratio (iii) glutamate/creatine ratio, (iv) glutamine/creatine ratio, (v) GABA/creatine ratio, (vi) GABA-glutamate ratio, (vii) taurine/creatine ratio and (viii) inositol/creatine ratio, The results are expressed as mean ± S.E.M. of 7-8 animals per group.

4. Frontal cortical synaptic density in methamphetamine-injected mice

METH did not modify synaptophysin and syntaxin-1 levels (pre-synaptic markers) in the frontal cortex 3 days post-injection when compared to controls as seen in Figure 20 ($p>0.05$).

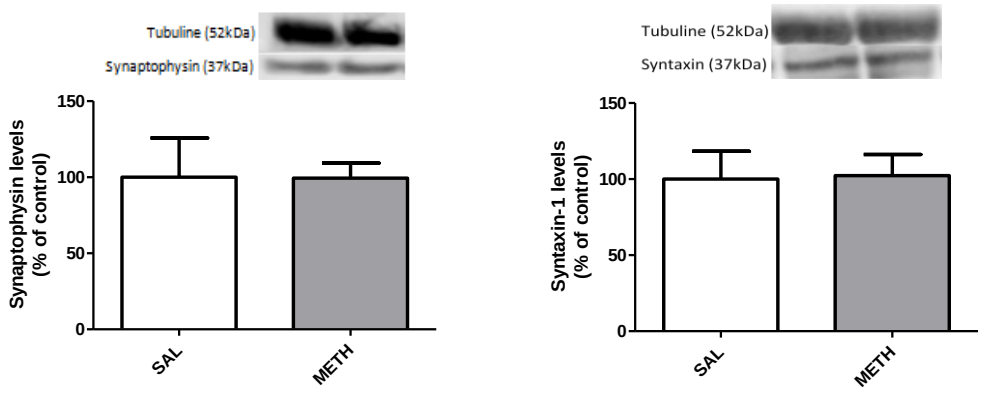

Figure 20 - Frontal cortical synaptophysin (A) and syntaxin-1 (B) levels from METH-and saline (SAL)-injected mice (measured by Western blot). All data are mean ± S.E.M, with 4-6 animals per group.

5. Frontal cortical levels of glucose transporter 1 ($GluT_1$) in methamphetamine-injected mice

METH did not modify both endothelial (55kDa) and astrocytic (45kDa) isoform Glut1 levels in the frontal cortex 3 days post-injection when compared to controls as seen in Figure 21 ($p>0.05$).

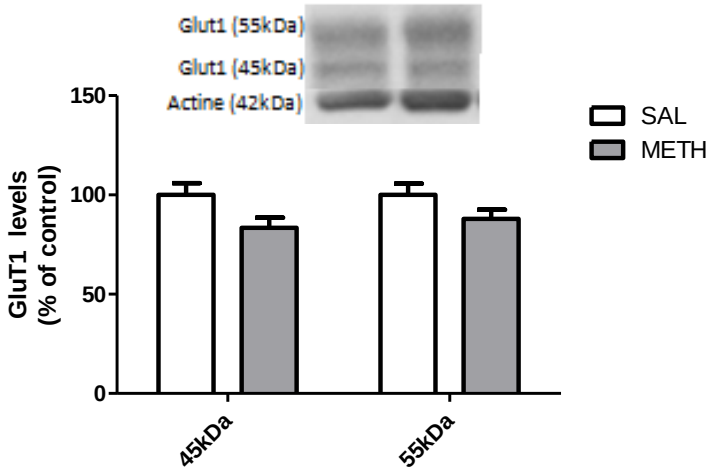

Figure 21 - Frontal cortical endothelial (55 kDa) and astrocytic (45 kDa) isoform Glut1 levels from METH-and saline (SAL)-injected mice (measured by Western blot). All data are mean ± S.E.M, with 4-6 animals per group.

Chapter 5

Discussion

Discussion

Herein we present novel data showing that a single high METH dose triggered an early anhedonia-like behavior. This negative emotional state is underlined by impaired frontal cortical energy metabolism as gauged by decreased NAA/Cre and Lac/Ala ratios measured by ^{1}H-NMR.

The METH regimen employed herein recapitulates the neurochemical and mood behavioural alterations seen in addicts after METH discontinuation (Krasnova and cadet, 2009; Silva et al., 2014; Rusyniak, 2013). The first objective of the current work was to further offer a comprehensive portrait of the negative emotional state of mice during early-METH withdrawal (72h post-injection). To this end we probed the anxiety- anhedonic- and despair-like behavior in METH-treated mice. Firstly, EPM test showed that the METH group did not exhibit an anxiety-like trait as gauged by similar amount of time and number of entries into open arms when compared to control animals. Furthermore by the METH group was not significantly different from the control group ($p>0.05$).

Two tests were used to assess depressive-like behaviour in METH-treated mice: the splash test and FST. However, only the splash test reported significant differences between groups – in fact, METH-injected mice showed decreased total grooming time. This is indicative of decreased self-care which is a read-out for an anhedonic-like behavior. This mimics apathy observed in depressed patients (Willner, 2005). This data further confirms the depressive-like behavior in mice evaluated 3 days post- similar METH dose by tail suspension test (Silva et al. 2014). However, the immobility time of METH-injected mice was not significantly different from control in the FST. This data further feeds existent controversy in the interpretation of this test: on the one hand, the immobility time has been classically interpreted as a behavioral correlate of negative

mood, representing a despair-like behaviour in the animal. However, there is some debate among the scientific community whether increased immobility demonstrates instead an adaptative learning i.e., the animal learned it cannot escape therefore it started to save energy until the experimenter removes it (Petit-Demouliere, Chenu, & Bourin, 2005). Also, this test is typically used to assess antidepressant efficacy, whereas TST is more sensitive in illustrating neurochemical abnormalities (Steru et al., 1985; Castagnne et al., 2010; Chatterjee et al., 2011). Overall, our findings are consistent with previous studies suggesting that rats experienced more depressive-like state than anxiety-like state during METH-early withdrawal using a self-administration model (Jang et al., 2013).

Spleen weight was controlled, as a stress indicator, since stress can result in a significant enlargement of the spleen, due to an increase in trafficking of CD11b+ myeloid cells from the bone marrow to the spleen (Engler et al., 2005). In the present study, no splenomegaly was reported, thereby, no stress was induced by the exposure to the drug.

The behavioral alterations prompted us to provide a metabolic explanation for this negative emotional state this negative. Thereby, a NMR-based metabolic mapping of METH-exposed mice allowed a general view over many brain metabolites during the early METH withdrawal. Using ^{1}H-NMR techniques, we detected profound metabolic changes in the levels of NAA, and the Lactate/alanine ratio, specifically at the prefrontal cortex.

These behavioral alterations prompted us to provide a metabolic explanation for this negative emotional state. Thereby, we are newly reporting a ^{1}H-NMR metabolomic fingerprint of both frontal cortex and hippocampus from mice experiencing early

METH- withdrawal. In fact, we disclosed metabolic changes in the levels of NAA/Cre, lactate/alanine ratio, at the prefrontal cortex.

NAA is one of the most concentrated brain metabolites and it usually produces one of the largest peaks in MRS scans (Moffett, Arun, Ariyannur, & Namboodiri, 2013). This metabolite is typically used as a surrogate marker of neuronal viability (Lan et al., 2008; Zhang et al., 2009). NAA alterations have been widely associated with several neurological and psychiatric disorders and substance-abuse conditions (Tsai & Coylet, 1990; Maddock & Buonocore, 2012). Moreover, NAA synthesis occurs primarily in the mitochondria (Patel and Clark, 1979) and reductions in its brain levels are paralleled by the reductions in ATP in several experimental paradigms illustrating brain energy metabolism impairment (Moffett et al., 2013). Therefore, decreased NAA levels seen herein are potentially consistent with compromised frontal cortex energetic in METH-treated mice.

Lactate is a product of glucose metabolism, through glycolysis, and it cannot be accumulated in astrocytes (Brooks, 2009). Therefore, it is shuttled to neurons trough the ANLS, and converted to pyruvate. Since pyruvate cannot be measured by MRS spectroscopy, we used alanine instead in the present work. The decrease in frontal cortical lactate/alanine ratio in METH mice may be indicative of a perturbed neuron-astrocyte metabolic coupling thus perturbing cortex energetics. However, this altered energy metabolism did no translate into hampered glutamate, glutamine and GABA (which is generated from glutamate) homeostasis. Our study offers a pioneer metabolomics analysis under a METH neurotoxic regimen. However, Bu et al. (2003) recently characterized metabolic rat brain alterations induced by subcutaneous injection of 2.5 mg/kg METH (twice-daily) for 7 days, using HR-MAS spectroscopy. These authors showed a decrease in frontal cortical and hippocampal NAA levels at the last

day of METH administration. Although the frontal cortical data is consistent with ours, the hippocampal data seems contradictory. Additionally, these authors also reported a depletion of glutamate, GABA, glutamine and taurine (oxidative stress marker) and an increase in inositol (glial marker) in both frontal cortex and hippocampus. However, one has to bear in mind that animal species (mice vs. rat), METH regimen (neurotoxic vs. sensitizing protocol) and evaluated time-points (withdrawal vs. acute effect) are different across studies. Therefore, strict comparison between studies warrants caution. Nonetheless, one might propose that at 3 days post-injection there was a substantial recovery of the metabolic perturbation, in both frontal cortex and hippocampus, except for the NAA and lactate frontal cortical levels. Notably, a restoration of perturbed metabolism after a 2-days withdrawal of METH was previously described, including the return of serum creatinine, citrate, 2-ketoglutarate and urinary lactate to baseline levels (Zheng et al., 2014).

No alterations were reported on the hippocampus, thereby, we focused our protein analysis on the PFC.

The next step was to evaluate whether energy impairment would translate into synaptotoxicity in frontal cortex from METH-injected mice. Syntaxin 1, along with synaptobrevin and synaptosome-associated protein (SNAP)-25 form a Ca^{2+}-dependent complex (SNARE complex) which precedes exocytosis and regulates the release of neurotransmitters from neurons (Edelmann et al., 1995). On the other hand, synaptophysin acts as a regulator of the SNARE complex (Hinz et al., 2001), and is also considered as a marker protein of presynaptic nerve endings (Thome et al., 2001; Grillo et al., 2005).

Syntaxin 1 and synaptophysin levels were not significantly different between saline and METH groups. While novel, this data is highly suggestive that there is no frontal cortical synaptotoxicity associated with METH at this time-point.

Recent data showing that 200μM METH – METH levels found in blood samples from chronic METH users - decreased both glucose uptake and $GluT_1$ levels in human astrocytes (Muneer et al. 2011) prompted us to analyze frontal cortical levels of $GluT_1$ Herein we demonstrated that METH failed to change both astrocytic (45kDa) and endothelial (55kDa) $GluT_1$ isoforms. However, one cannot rule out that glucose uptake could be playing a role on the defective neuron-astrocytic metabolic coupling seen herein.

Chapter 6

Conclusion

Conclusion

The present study presents novel data showing that a single neurotoxic methamphetamine dose (30mg/kg), triggered an early anedhonic-like state accompanied by frontal cortical energy metabolism disruption.

Further studies are warranted to analyze the glucose uptake and lactate release using frontal cortical slices isolated from METH-treated mice.

Chapter 7

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