Exploring new possible targets in Methamphetamine exposure

Joana Alexandra Moreira Ribeiro
Mestrado em Biologia Celular e Molecular
Departamento de Biologia
2016

Orientador
Teresa Summavielle, PhD
Coorientador
Andrea Lobo, PhD

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Agradecimentos

Agradeço, em primeiro lugar, à Doutora Teresa Summavielle por me ter recebido de portas abertas e por me ter permitido ficar. Obrigada por me ter orientado e ajudado em tudo o que precisei.

Agradeço à Andrea tudo o que me ensinou e por toda a paciência com que o fez. Obrigada pelas ideias e pela orientação.

Ao Renato agradeço a ajuda nos momentos mais difíceis. Agradeço por resolver sempre os problemas entre a cadeira e o computador. Obrigada por todos os bons momentos e por tudo o que me ensinaste.

À Teresinha, à Renata e ao João, que me ajudaram em tudo o que puderam. E mesmo quando não podiam, arranjavam forma. Obrigada pelos bons momentos e por toda a idiotice. Todos me ensinaram mais do que aquilo que merecia, tanto dentro como fora do laboratório. Obrigada pela paciência com os meus dramas e com os meus erros. Vocês tornaram este ano memorável.

A todas as outras pessoas do laboratório agradeço o apoio e as palavras amigas.

À minha gente: Ana João, Filipa, Márcia, Maria, Sandra, Teresa, Vanessa e Jorge. Comecei esta aventura há cinco anos ao vosso lado e vou acabar ao vosso lado. Não consigo dizer-vos quão importantes são para mim. Estes últimos anos foram incríveis e foi graças a vocês. Obrigada por tudo, por tudo mesmo.

Quero também agradecer à Zezinha por ter sido um rochedo na altura mais difícil da minha vida. Sem ela nunca teria chegado até aqui.

Por último, agradeço à minha família. Fizeram de mim quem sou e estiveram sempre disponíveis para mim. Aos meus pais por me darem todo o amor, carinho e paciência, mesmo quando eu não merecia. Por sempre me incentivarem a perseguir os meus sonhos e por me darem liberdade para o fazer. Ao meu tio, sempre presente, obrigada por ser a calma na tempestade, mesmo quando parecia não haver solução. À minha priminha Catarina, que é família de sangue e família da alma. Esteve sempre lá, no bom, no mau e no muito mau. Obrigada por resmungares comigo quando foi preciso e obrigada pelas conversas estúpidas das duas da manhã que nenhuma de nós compreende!

Este trabalho é dedicado aos meus avós que tanto fizeram por mim. Sei que estariam orgulhosos.

Resumo

A metanfetamina (METH) é uma droga psicostimulante, altamente aditiva e uma das mais utilizadas em todo o mundo. A dependência de drogas é considerada uma doença cerebral crónica, onde as recaídas são frequentes, associada a doenças sistémicas graves, o que levanta preocupações socioeconómicas e de saúde pública relevantes. Embora indivíduos dependentes geralmente apresentem condições fisiopatológicas graves, o tratamento da toxicodependência é atualmente escasso e os mecanismos pelos quais a dependência se desenvolve não são ainda completamente conhecidos. Assim, é essencial explorar estes mecanismos, a fim de encontrar possíveis alvos terapêuticos. A METH, em particular, conduz a uma disfunção de longo prazo de neurónios dopaminérgicos e glutamatérgicos, comprometimento de células da glia e *stress* oxidativo elevado. A exposição a psicostimulantes também afeta a morfologia neuronal, o aumento do comprimento de neurites e a densidade de espículas dendríticas em várias regiões do cérebro.

Para explorar esta questão, realizamos uma análise de *RNA-Seq* em frações de soma e de neurites de neurónios do hipocampo em cultura, o que revelou padrões de expressão diferenciados em vários genes em neurónios expostos a METH. Inicialmente, selecionamos genes expressos diferencialmente no soma, relacionados com o metabolismo, a sinalização celular, o ciclo celular ou transcrição/tradução. Foram selecionados 5 genes que poderiam estar relacionados com a neuroinflamação e interação neurónio/glia: *Optineurin* (Optn), *Olfactomedin 4* (Olfm4), *RAS guanyl releasing protein 1* (Rasgrp1), *RelB Proto-Oncogene* (RelB) e *Early B-cell factor 3* (Ebf3).

Realizou-se *RT-qPCR* para validar a expressão dos candidatos selecionados, e confirmou-se que a exposição a METH aumenta significativamente os níveis de expressão de mRNA de *Olfm4* em culturas primárias de neurónios do hipocampo. A expressão proteica foi avaliada através de *western blot* e imunocitoquímica, revelando que os níveis de proteína Olfm4 estão diminuídos. Esta é uma proteína da matriz celular conhecida por interagir com integrinas e metaloproteínases para além de poder estar envolvida na regulação da neuroinflamação, como tal é alvo de interesse no contexto da exposição a drogas psicoestimulantes.

Analisámos assim a influência da METH na expressão de fatores inflamatórios através de *RT-qPCR*, embora sem sucesso. Além disso, avaliamos o efeito da METH e da proteína Olfm4 em proteínas relacionadas com a matriz extracelular (*Cldn5*, *ZO-1*, *MMP2*, *Src* e *RhoA*) através de análises de *western blot*, imunocitoquímica, zimograma e FRET. Mostramos que a METH não afeta a expressão da *Cldn5* e *ZO-1*. A exposição a METH não parece afetar significativamente a atividade da *MMP2*, levando no entanto a um aumento na

ativação da *Src*, e diminuição da ativação da *RhoA*. A ativação da *Src* parece estar também associada à expressão da *Olfm4*.

No geral, não observamos efeitos da METH na expressão de proteínas relacionadas com a matriz extracelular em neurónios em cultura, nem evidência de contribuição para processos de neuroinflamação. No entanto, identificámos o envolvimento da METH na ativação da *Src,* o que associado à consequente inativação da *RhoA*, pode contribuir para as alterações morfológicas neuronais induzidas pela METH.

Palavras-chave: Fatores Inflamatórios; Metanfetamina; Neurónios do Hipocampo; Remodelação da Matriz

Abstract

Methamphetamine (METH) is a highly addictive psychostimulant drug and one of the most abused worldwide. Drug addiction is considered a chronic and relapsing brain disease associated to severe systemic disorders, which raises relevant socio-economic and public health concerns. Although addicted individuals usually present severe pathophysiological conditions, addiction treatment is currently scant and the mechanisms by which addiction develops are still elusive. Therefore it is essential to explore these processes in order to find new possible therapeutic targets. METH in particular, leads to long-term dysfunction of dopaminergic and glutamatergic neurons, glial impairment and high oxidative stress. Interestingly, exposure to psychostimulants also affects neuronal morphology, increasing neurite length and dendritic spine density in several brain regions.

To explore this issue, we conducted an RNAseq analysis from both soma and neurite fraction of cultured hippocampal neurons, which revealed differential expression patterns in numerous genes in METH exposed neurons. In a first step, we have selected genes differentially expressed in the soma, related to metabolism, cell signaling, cell cycle or transcription/transduction. We selected 5 genes that were likely to be related to neuroinflammation and neuron/glia interplay: Optineurin (Optn), Olfactomedin 4 (Olfm4), RAS guanyl releasing protein 1 (Rasgrp1), RelB Proto-Oncogene (RelB) and Early B-cell factor 3 (Ebf3).

We conducted RT-qPCR to validate the expression of the selected candidates, and confirmed that upon exposure to METH the Olfm4 mRNA expression levels were significantly increased in primary cultures of hippocampal neurons. Protein expression was evaluated through western blot and immunocytochemistry, revealing that Olfm4 protein levels are decreased. Olfm4 is known to interact with integrins and metalloproteinases and has been linked to inflammatory processes.

We have also analysed METH influence in inflammatory factors expression through RT-qPCR, but found no evidence of increased expression of neuroinflammatory factors. Moreover, we have assessed METH effect on extracellular matrix related proteins (Cldn5, ZO-1, MMP2, Src and RhoA) by western blot, immunocytochemistry, zymography and FRET assays. We show that METH does not affect Cldn5 or ZO-1 expression. Also, METH exposure does not appear to significantly affect MMP2 activity in neuronal cells, leading however to an increase in Src activation, while decreasing RhoA activation. Interestingly, Src activation appears to decrease when Olfm4 is knocked down.

Overall, we did not observe an effect of METH in the expression of extracellular matrix related proteins in neuronal cells, nor evidence of its contribution for the neuroinflammatory process described for METH in humans and in vivo models. However, we

might have uncovered a new pathway, involving Src activation and consequent RhoA inactivation, which may contribute to the METH-induced neuronal morphological alterations.

Keywords: Hippocampal neurons; Inflammatory Factors; Matrix Remodeling; Methamphetamine

Índex

List of tables and figures

Tables

Figures

Abbreviations

5-HTT Serotonin Transporters

ADHD Attention Deficit Hyperactivity Disorder

BBB Blood-Brain Barrier

CNS Central Nervous System

DAT Dopamine Transporters

DEA Drug Enforcement Administration

DIV Days in Vitro

Dnmt1 DNA (Cytosine-5)-Methyltransferase 1

Dnmt2 DNA (Cytosine-5)-Methyltransferase-Like Protein 2

ETC Electron Transport Chain

ER Endoplasmic Reticulum

G-CSF Granulocyte Colony-Stimulating Factor

GLU Glutamate

HBSS Hank's balanced salt solution

IL-1β Interleukin 1β

IL-6 Interleukin 6

MAO monoamine oxidase

METH Methamphetamine

MMP2 Matrix metallopeptidase 2

MMP9 Matrix metallopeptidase 9

NAc Nucleus Accumbens

NET Noradrenaline Transporter

NMDAR N-methyl-D-aspartate Receptor

NOS Nitric Oxide Synthase

PU.1 Transcription factor PU.1

RNS Reactive Nitrogen Species

ROS Reactive Oxygen Species

SERT Serotonin Transporter

SN Substantia Nigra

TNF Tumor Necrosis Factor

VMAT-2 Vesicular Monoamine Transporter-2

VTA Ventral Tegmental Area

Ywhaz Tyrosine 3-Monooxygenase

Chapter 1: Introduction

1.1. Drug addiction

Drug addiction is a neurobiological, chronical disorder with a persistent possibility of relapse. This disorder is characterized by compulsion to seek and take drugs, loss of control in limiting intake and development of a negative emotional state when the access to drug is prevented. The repetitive drug abuse impairs the normal circuitry of rewarding leading to drug-induced neuroplastic changes (Arias-Carrión et al, 2010; Koob and Le Moal, 2005).

Addicted individuals tend to ignore the negative consequences of drug intake and become focused on obtaining and taking drugs (Hyman et al, 2006). The occasional use of a drug (with the potential for abuse or dependence) is distinct from heightened drug use and the development of a chronic drug-dependent state (Koob, 2006). In fact, only a small percentage of individuals exposed to drugs will become addicted, that is, go from controlled drug use to compulsive use, despite the adverse consequences. Genetic (50% risk), developmental (higher risk in adolescence) and environmental factors (e.g., drug access, stress), as well as the type of drug used, are among the causes the rule the propensity of an individual to become an addict (Volkow and Li, 2005).

During the transition from recreational use to addiction, a motivational shift takes place. A drug is no longer taken to obtain pleasure from it, but, instead it is taken to satiate the intense craving and to relieve the distress of not having access to it (Volkow and Li, 2004).

Drug addiction yields a cycle composed of three stages: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation (craving). Impulsivity often dominates at the early stages, and impulsivity combined with compulsivity dominates at the later stages (Koob and Volkow, 2010).

1.2. Methamphetamine

Methamphetamine (METH), also known by its “street names” as ice, crank, speed or crystal (among others), is a synthetically derived psychostimulant drug that acts on the central nervous system (CNS) (figure 1). It presents significant abuse potential and neurotoxic effects. Due to its small size and lipophilicity, METH can easily cross the blood-brain barrier (BBB) and acts mostly by causing the release of central and peripheral monoamines. It can be synthesized in a straightforward one-step process by reduction of ephedrine or pseudoephedrine, or by condensation of phenylacetone and methylamine. Being a simple reaction, it only requires rudimentary laboratory equipment to produce (Barr

et al, 2006; Cho, 1990; Gold et al, 2009; Nordahl et al, 2003; Panenka et al, 2013; Turowski and Kenny, 2015).

The inexpensive production of METH, together with the low cost and long lasting effect of the drug are the main aspects that make METH consumption appealing (reviewed in Krasnova and Cadet, 2009). In spite of its addictive properties, oral METH continues to be used in the USA as a treatment for various medical conditions, including attention deficit hyperactivity disorder (ADHD) and refractory obesity (Kish, 2008).

METH is typically ingested, smoked, snorted or injected intravenously (Nordahl et al, 2003). The drug metabolism occurs mostly in the liver, leading to the production of numerous metabolites (Caldwell et al, 1972).

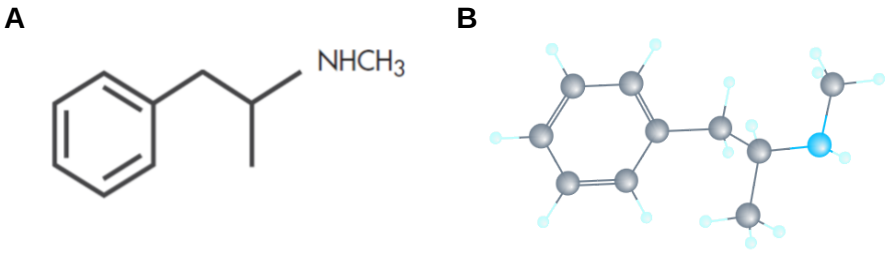


Figure1. Methamphetamine. Chemical diagram (A) (adapted from Nordahl et al, 2003) and tridimensional METH conformation (B) (adapted from PubChem).

1.3. Historical perspective

METH was first synthetized in 1893 by Nagayoshi Nagai, a Japanese pharmacologist. However, METH use only became widespread during World War II, when USA, Germany and Japan made it available to their soldiers in order to enhance their performance. When the war ended, surplus army stocks invaded the market and the civilian use increased (Anglin et al, 2000; Weisheit and White, 2009). From the 1930s onwards, amphetamines were prescribed for a range of medical conditions, including weight loss, treatment of fatigue, depression and narcolepsy (EMCDDA, 2014).

In 1971, the United Nations Convention on Psychotropic Substances declared amphetamines and METH schedule II drugs (Final Act of the United Nations Conference for the Adoption of a Protocol on Psychotropic Substances, 1971). According to Drug Enforcement Administration (DEA), schedule II drugs are substances, or chemicals, with a high potential for abuse, which use probably leads to severe psychological or physical dependence. Since then, amphetamines manufacturing and sale became forbidden in most European countries (EMCDDA, 2014).

Nowadays, amphetamine production is mostly concentrated in North America and Asia-Pacific regions. Accordingly, from a total of 144 tons of amphetamine-type stimulants seized globally in 2012, half were seized in North America and a quarter in East and South-East Asia (World Drug Report, 2014). Since mid-2000s, METH production has spread to places like South Africa or Iran. Production in new world regions may influence drug markets in Europe, in response to new buyers (European Drug Report 2014: Trends and developments, 2014).

The number of amphetamine type stimulants-manufacturing laboratories dismantled increased from 12571 in 2011 to 14322 in 2012, being 96% of those manufacturers of METH (World Drug Report, 2014; figure 2).

METH and amphetamine use has been reported in Europe, representing a major problem in some specific countries like Czech Republic and Slovakia (Problem amphetamine and methamphetamine use in Europe, 2010; figure 2).

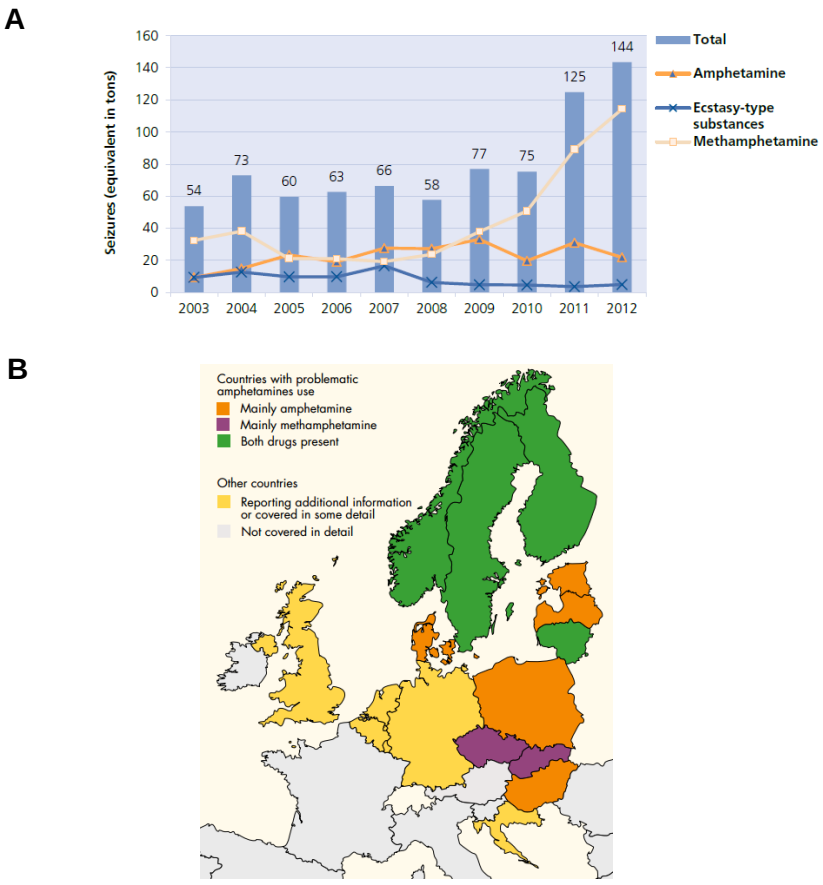


Figure 2. Amphetamines seizures and usage. Global seizures of amphetamine-type stimulants from 2003 to 2012 (A) and use of amphetamines in Europe (B) (adapted from World Drug Report 2014 and EMCDDA, 2010).

1.4. Clinical effects

Clinically, METH induces physiological changes similar to those produced by the fight-or-flight response. These include the increase of blood pressure, body temperature, heart and breathing rate (Anglin et al, 2000). Feelings of euphoria, increased productivity, hypersexuality and energy have also been described (Homer et al, 2008; Meredith et al, 2005). Other effects include acute toxicity, altered behavioral and cognitive functions, as well as impaired judgment, euphoric disinhibition, memory loss and neurological damage. Users might also experience agitation, aggressiveness, tachycardia, hypertension and hyperthermia (Albertson et al, 1999; Barr et al, 2006; Meredith et al, 2005; Lynch and House, 1992; Murray, 1998; Scott et al, 2007; Thompson et al, 2004; reviewed in Krasnova and Cadet, 2009).

All of these effects may last for several hours, since METH's half-life varies between 10 to 12 hours (Schepers et al, 2003).

Larger doses of METH consumption can lead to life-threatening hyperthermia (above 41°C), renal and liver failure, cardiac arrhythmias, heart attacks, cerebrovascular hemorrhages, strokes and seizures (Albertson et al, 1999; Darke et al, 2008; Perez et al, 1999). On the other hand, withdrawal can lead to anhedonia, irritability, fatigue, impaired social functioning and intense craving for the drug (Brecht et al, 2004; Darke et al, 2008; Homer et al, 2008; Sekine et al, 2006; Zweben et al, 2004).

At the molecular level, METH causes neurodegenerative changes like persistent decreases in the levels of dopamine transporters (DAT) in the orbitofrontal cortex, dorsolateral prefrontal cortex and the caudate–putamen (McCann et al, 1998; McCann et al, 2008; Sekine et al, 2003; Volkow et al, 2001a,c; reviewed in Krasnova and Cadet, 2009). It is also possible to verify a decline of serotonin transporters (5-HTT) in the midbrain, caudate, putamen, hypothalamus, thalamus, the orbitofrontal, temporal and cingulate cortices (Sekine et al, 2006). Abnormal glucose metabolism may be present in cortical and subcortical brain areas (Volkow et al, 2001b; Wang et al, 2004).

Noticeable microglial activation is present in the midbrain, striatum, thalamus, orbitofrontal and insular cortices of METH abusers. These activated microglia release pro-inflammatory cytokines, which can both stimulate glutamate (GLU) release and inhibit its uptake. This contributes to an increased activity of nitric oxide synthase (NOS) and reactive nitrogen species (RNS) production, and also reactive oxygen species (ROS). The activated microglia acts as the immune cells of the brain and contributes to the neuroinflammation induced by METH (discussed in more detail later in this chapter) (reviewed in Marshall and O'Dell, 2012; Sekine et al, 2008).

1.5. Methamphetamine action mechanism

METH action on the CNS is related to its role as a monoaminergic agonist, affecting simultaneously the dopamine, noradrenaline and the serotonin transport (reviewed in Cruickshank and Dyer, 2009). Under physiological circumstances, monoamines are stored in synaptic vesicles by the action of vesicular monoamine transporter-2 (VMAT-2) and released into the synaptic cleft upon neuronal activation. They are then transported back into the nerve terminal by membrane transporters (DAT, noradrenaline transporter (NET), the serotonin transporter (SERT)) and recycled back into synaptic vesicles. METH reverts the functioning of DAT, NET, SERT and VMAT, becoming a substitute for dopamine, noradrenaline and serotonin (Sulzer et al, 2005), therefore interfering with monoaminergic signaling (Fleckenstein and Hanson, 2003; Masson et al, 1999; Pacholczyk et al, 1991). This leads to dysfunction in these circuitries, which increases with high-doses and repeated administration resulting in persistent monoaminergic deficits (particularly, dopamine deficits) (reviewed in Riddle et al, 2006).

METH reversion of the membrane transporters function, not only allows METH to enter the pre-sypnatic terminal, but also results in increased release of monoamines from the cytosol to the synaptic cleft while also decreasing monoamine metabolism by inhibiting monoamine oxidase (MAO), promoting increased stimulation of postsynaptic monoamine receptors (Sulzer et al, 2005).

METH also generates an increase in the GLU release. The increased extracellular GLU activates NMDAR (N-methyl-D-aspartate receptor), which results in a higher intracellular Ca^{2+} influx. This increased intracellular Ca^{2+} activates enzymatic pathways that will cause a rise in the production of ROS and RNS (reviewed in Marshall and O'Dell, 2012; reviewed in Riddle et al, 2006).

METH consumption leads to an unbalance in the mechanisms required for normal brain functioning, mainly due to DAergic deficit. METH's lipophilicity may contribute for it to diffuse through cell and organelles membranes, contributing to inhibition of normal mitochondrial functioning. This results in decreased energy and increased reactive species production. The electron transport chain (ETC) does not work properly, resulting in an insufficient ATP production and a higher leakage of high energy electrons, which contributes to ROS production. The immense production of ROS and RNS has a prejudicial effect on neurons, causing their degeneration (reviewed in Marshall and O'Dell, 2012).

Figure 3 represents METH induced effects at the synapse, affecting neurons, glia and endothelial cells.

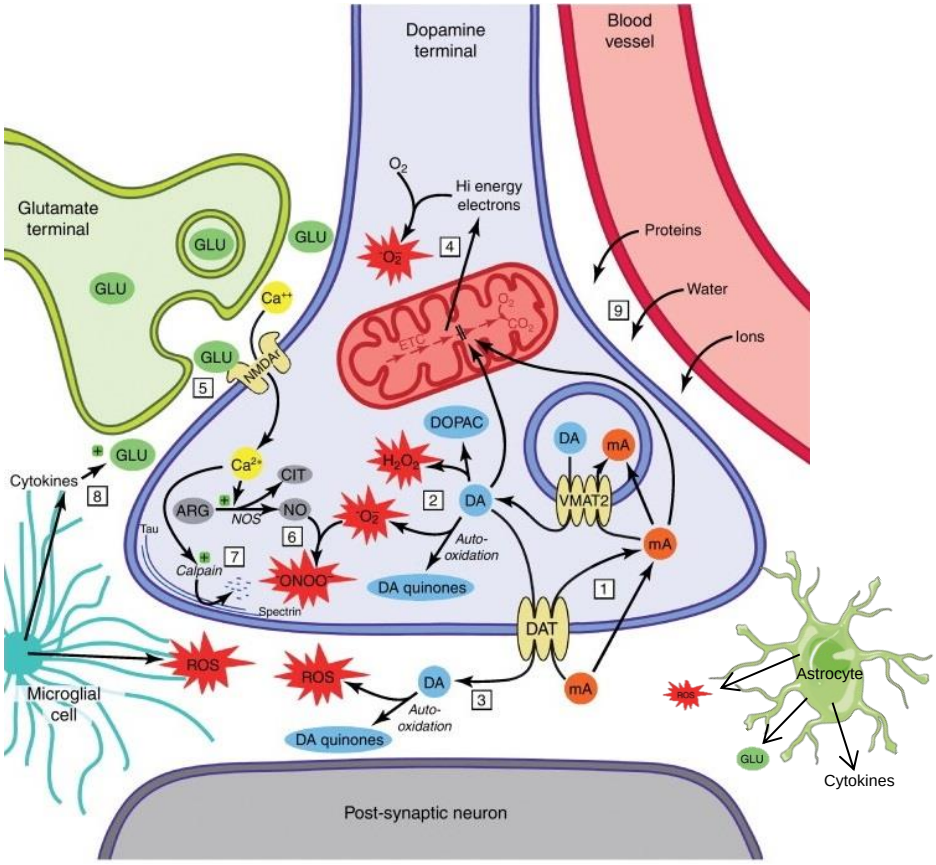


Figure 3. Mechanisms of methamphetamine neurotoxicity. METH enters dopaminergic terminals **(1)**, causing efflux of DA. DA metabolism produces reactive species **(2)** that are transported to extracellular spaces **(3)** where they are oxidized producing ROS. High intracellular concentrations of DA and METH can inhibit ETC in mitochondria **(4)**, causing leakage of high-energy electrons resulting in formation of superoxide. METH-induced increases in GLU release **(5)** stimulate NMDAR on dopaminergic terminals leading to increases in intracellular Ca^{2+} that stimulates NOS activity, increasing the production of nitric oxide (NO) that may combine with superoxide to form peroxynitrite ($^{\cdot}ONOO^{-}$) **(6)**. High intracellular Ca^{2+} can also induce proteolytic enzymes (e.g.calpain) **(7)** that break down structural proteins (e.g.spectrin) resulting in impaired terminal integrity. Furthermore, microglia releases ROS and cytokines **(8)**, which contributes to increase extracellular GLU levels. METH causes BBB impairment, allowing plasma proteins to enter the brain **(9)**, followed by water and ions, causing brain edema and additional physiological disruption of neurotransmission (adapted from Marshall and O'Dell, 2012).

1.6. Methamphetamine and the reward system

The brain's main function is to assure individual survival and gene propagation. In order to survive, individuals need to acquire substances for their bodily functions (namely foods and liquids) as well as adopting certain crucial behaviors (e.g., sexual behavior for reproduction or hiding from predators for survival), which require effort to obtain or perform (Schultz, 2010; Schultz, 2013). These substances and/or behaviors are called rewards and support learning, decision making and positive emotions like pleasure, desire and happiness (Schultz, 2013).

Rewards can be roughly divided into three components: liking, wanting and learning. Liking represents the actual pleasure impact of a reward; wanting links to the motivation for the reward, and learning is related with associations, representations and predictions about

future rewards based on past experiences. Rewards can lead to either a conscious or to an unconscious response (Berridge and Kringelbach, 2008).

Drugs use is initiated and sustained in large part by their hedonic (i.e., pleasurable) effects. Indeed, all major drugs of abuse activate the brain reward system, providing major motivation to obtain and consume addictive drugs (Kenny, 2007; Nesse and Berridge, 1997). In the beginning, liking the drug will be a sufficient motivation to use it. With time, there will be a shift in this motivation: a drug won't be liked; it will be wanted, thus contributing to addiction (Robinson and Berridge, 2008). METH is a reward-related drug, which is why it is consumed by humans or self-administered by laboratory animals (Wise, 2009). There are different kinds of reinforcement that contribute to compulsive abuse of drugs. Positive reinforcement occurs when presentation of the drug increases the probability of a response to obtain the drug again. On the contrary, negative reinforcement occurs with mitigation of an existing aversive state or a drug-generated aversive state (e.g., withdrawal) (reviewed in Koob and Le Moal, 2001).

The rewarding effects of drugs are caused by their ability to increase DA release in the nucleus accumbens (NAc) (Volkow et al, 2012). The amount of DA delivered to the NAc is directly involved in the hedonic component of reward (reviewed in Phillips et al, 2008). Resorting to behavioral studies, Schultz (2007) showed that DA projections to the striatum and frontal cortex also play a central role in mediating the effects of rewards on approach behavior and learning, providing evidence for a possible important role in learning and memory processes.

After experiencing reward, an individual has the motivation to seek that same reward again. Environmental cues associated to a specific reward are meaningful reinforcing associations between reward and stimuli linked to that same reward. These cues are usually sufficient to initiate DA release by reward anticipation (Arias-Carrión et al, 2010). Concerning drug reward, the mesolimbic DA pathway [DA neurons in ventral tegmental area (VTA) projecting into NAc] appears to be the most relevant one (Wise, 2009). However, recent studies recognize the mesostriatal DA pathway [DA neurons in substantia nigra (SN) projecting into dorsal striatum] and the mesocortical DA pathway (DA neurons in VTA projecting into frontal cortex) to contribute to drug reward and addiction as well (Wise, 2009; 3).

Moreover, it is also known that D1 and D2-like receptors, respectively excitatory and inhibitory receptors for DA, are stimulated by amphetamines and might be involved in drug-induced effects. D1 receptors are stimulated by large DA increases and are coupled to the Gs proteins, which activate adenylyl cyclase. D2 receptors are stimulated by relatively low DA concentrations and are coupled to Gi proteins, which inhibit adenylyl cyclase and activate

K^+ channels. Both receptor families are implicated in drug reward (Durieux et al, 2009; Missale et al, 1998; Vallone et al, 2000).

Glutamatergic neurotransmission is also related with drug addiction. An increase in extracellular GLU might lead to excitotoxicity, due to increased activation of NMDAR and the consequent increase of intracellular Ca^{2+}. METH induces increase of extracellular GLU in brain regions such as the VTA, the NAc, the prefrontal cortex and the striatum (reviewed in Cunha-Oliveira et al, 2008). It is believed that GLU modulates the reactivity of dopaminergic cells and dopamine release in NAc, regarding the reward effects of drugs of abuse (Kalivas and Volkow, 2005; figure 4).

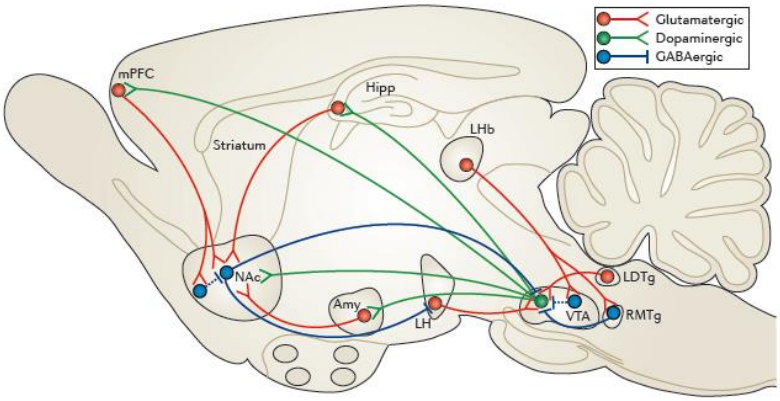


Figure 4. VTA–NAc reward circuit in rodents brain. Dopaminergic, glutamatergic and GABAergic connections to and from the VTA and NAc. Reward circuit includes dopaminergic projections from VTA to NAc, which release dopamine in response to reward-related stimuli, and GABAergic projections from NAc to VTA. Glutamatergic inputs control aspects of reward-related perception and memory. The dashed lines indicate internal inhibitory projections (adapted from Russo and Nestler, 2013).

1.7. Neuroinflammation associated to methamphetamine use

As mentioned above, METH is capable of crossing the BBB and inducing its disruption. The CNS, when exposed to METH, shows damage in the BBB across all of its extent. However, hippocampus appears to be more sensitive to this deterioration, which causes proteins and ions to leak into the brain causing water to osmotically enter the brain parenchyma, generating brain edema. BBB disruption might involve METH-induced ROS production and hyperthermia, both of which are described to trigger BBB breakdown (Bowyer et al, 2008; reviewed in Marshall and O'Dell, 2012; Martins et al, 2011; Montagne et al, 2015; Sharma and Kiyatkin, 2009; Terrando et al, 2014).

Inflammation is a response that has the purpose of repairing damages inflicted by a harmful agent (e.g., infection or traumatism). It is a complex response that involves cells,

plasma components and cellular products (Chavarria and Alcocer-Varela, 2004). It implicates the synthesis and release of proinflammatory mediators like cytokines and chemokines (O'Callaghan et al, 2008). Inflammation is considered a part of the innate immune system since it is a generic response to harmful stimuli (Abbas et al, 2012).

In the CNS, microglial cells are involved in immune surveillance and are activated during neurodegenerative processes. They are recognized as being one of the fundamental components of an intrinsic brain immune system and are the major antigen-presenting cells in the brain. When activated by tissue injury, they release signaling molecules involved in inflammation, which may lead to neuronal damage. Moreover, microglia participates in shaping neuronal connectivity during development and in activity-dependent synaptic plasticity, neurogenesis, and learning (Kreutzberg, 1996; reviewed in Loftis and Janowsky, 2014; reviewed in Salter and Beggs, 2014; Streit and Kincaid-Colton, 1995).

Pro-inflammatory signaling is often involved in the dysfunction of the BBB's structural and functional integrity (Abbott et al, 2006; Persidsky et al, 2006) and the inflammatory response by resident neuroglia, especially microglia, perpetuates BBB dysfunction, which in turn will increase the neuroinflammatory response (Petty and Lo, 2002). Disruption of the BBB caused by high-dose METH exposure is associated with neurodegeneration and activation of brain microglia and/or infiltration of macrophages. This will lead to immune dysfunction, with increased leukocyte/monocyte transmigration across the endothelium, and into the CNS, as well as increased invasion of peripheral bacteria and viruses into the brain (Buch et al, 2012; reviewed in Loftis and Janowsky, 2014; Ramirez et al, 2009).

There are several studies reporting microglia activation in response to METH. For instance, Sekine et al (2008) described microglia activation in brains of METH addicts, especially in regions of dopaminergic and serotoninergic innervation. Gonçalves et al (2009) also reported microglia and astrocytes activation in the mouse hippocampi in response to METH administration. This activation is dose-dependent and may lead to an alteration of the peripheral and central immune functions (reviewed in Loftis and Janowsky, 2014; Thomas et al, 2004).

It is also known that neurons are capable of producing inflammatory mediators and actively control microglia activity. Endangered or stressed neurons appear to emit signals of cytosolic or membrane origin leading to microglia activation (Streit, 2002). Neurons express "On" and "Off" signals to inform microglia about their status: "Off" signals are constitutively found in neurons and work by disappearance, meaning that when their production stops, microglia gets activated; whereas "On" signals are produced on demand and operate by appearance, in other words, their presence induces microglia activation. This new evidence shows that neurons, although vulnerable, may also have a rather active role in modulating mechanisms of microglial activation to maintain CNS integrity (reviewed in Biber et al, 2007).

Even slight alterations in the CNS homeostasis inform microglia, allowing these to provide defense against infection, injury and disease (Jurgens and Johnson, 2012).

CNS immune mechanisms seem dependent of neuronal health and activity, indicating the importance of neurons in the regulation of immune cells (reviewed in Biber et al, 2007; Levite, 2008). Mott et al (2004) demonstrated that neurons must have functional axon terminals to inhibit microglia activation, pointing that physiologically intact neurons are necessary for regulating this activation. Besides, microglial cells express various receptors for neurotransmitters, neuropeptides and neuromodulators, showing that they have the capacity to sense neuronal activity (Pocock and Kettenmann, 2007).

Moreover, METH-induced GLU release and, therefore, activation of GLU receptors results in a higher production of proinflammatory cytokines interleukin 1β (IL-1β), tumor necrosis factor (TNF) and interleukin 6 (IL-6). These cytokines can increase extracellular GLU levels by inhibiting GLU uptake or increasing GLU release from activated microglia, resulting in a cycle that contributes to neurotoxicity (reviewed in Yamamoto et al, 2010).

In summary, it is known that microglia and astrocytes activation is a normal reaction to brain injury. However, excess neuroinflammation can lead to further brain damage. Repeated or high dose METH exposure induces alterations in glial and neuronal cell functions, which will contribute to a cascade of events, involving the production of cytokines and chemokines, and leading to neuroinflammation, neuronal damage and behavioral impairments (reviewed in Loftis and Janowsky, 2014). Neuroinflammation has a series of consequences in neurons, ranging from synaptic dysfunction to neuronal death. Beyond these, neurogenesis might become impaired which might contribute to dementia. Cognitive impairment is another consequence of neuroinflammation, since it has been described that both short and long term memory are affected by inflammatory factors (reviewed in Lyman et al, 2014). Neuroinflammation is also associated with neurodegenerative diseases such as Alzheimer’s disease, Parkinson's disease, Huntington's disease and multiple sclerosis, among others (Shabab et al, 2016). Furthermore, Narita et al (2006) has described that astrocyte activation in response to METH exposure results in the modulation of the reward mechanism, contributing for the rewarding effects caused by the drug.

1.8. Influence of methamphetamine on gene expression

The studies reporting METH-induced alterations on gene expression are abounding and diverse. The overall notion is that METH modifies gene expression, which will have consequences on cells and organisms. The study performed by Numachi et al (2004), for instance, reveals METH’s effect on DNA (Cytosine-5)-Methyltransferase-Like Protein 2 (Dnmt2) mRNA in the hippocampus, which becomes decreased 24 hours after treatment.

The study mentions a previous work where it was also observed that DNA (Cytosine-5)-Methyltransferase 1 (Dnmt1) mRNA in the hippocampus is decreased, 3 hours after acute METH treatment. Dnmt1 is a maintenance methyltransferase, involved in maintaining the preexisting methylation pattern of DNA, while Dnmt2 is involved in DNA methylation and might be involved in DNA repair, DNA recombination and carcinogenesis (Numachi et al 2004). METH appears to be involved in chromatin modulation through epigenetic pathways (e.g. ΔFosB) which may contribute to amphetamine-induced neuronal plasticity and associative learning while also regulating sensitivity to repeated drug exposure (Kalda et al, 2007; Renthal et al, 2008).

A number of genes related to trafficking and protein turnover, metabolic pathways, transmitters, receptors and growth factors (cytokines) were shown to be upregulated in response to METH treatment. On the other side, some genes related to trafficking and protein turnover, modulators, effectors and intracellular transducers were decreased (Asanuma et al, 2004). Nikaido et al (2001) investigation revealed that expression of genes involved in circadian rhythms increased in the caudate-putamen of mice in response to acute METH treatment. Moreover, there is evidence of METH-induced activation of NF-κB, upregulation of TNF, increase of the expression and activity of matrix metallopeptidase 9 (MMP-9) and reduction of tight junction proteins expression (Fernandes et al, 2014; Lee et al, 2001; Martins et al, 2011; Ramirez et al, 2009).

1.9. Objectives

The understanding of the molecular mechanisms underlying METH neurotoxic effects will likely contribute to provide new therapeutic approaches. Having that in mind, we have recently conducted a RNA deep sequencing analysis to identify altered gene expression in both soma and neurites of hippocampal neurons exposed METH. The present research project starts at that point, analyzing the results of the RNAseq and aiming to identify genes that may become useful targets for future therapies. In particular, we have focused on genes that could be associated with the neuroinflammatory process that underlies addiction, since we have evidence that neuroinflammation may be associated with poor success in treatment and withdrawal attempts and may potentiate relapse. Therefore, we have selected genes involved in the neuron-glia interplay and have attempted to dissect the role of these candidates in METH-induced neuroinflammatory pathways.

Our specific goals were:

i) Select the candidates of interest related to neuroinflammatory pathways and neuron-glia interplay, which expression was shown to be altered in the soma of hippocampal neurons by METH administration;

ii) Evaluate gene and protein expression of the selected candidates, and select one candidate for further studies;

iii) Functionally evaluate the role of the selected candidate, by modulating it expression in the presence of METH.

Chapter 2: Methodology

2.1. Hippocampal neuronal cells culture

The number of mice handled for this research was approved by the Institutional and National General Veterinary Board Ethical Committees according to National and European Union rules. Three- to six-month-old C57Bl/6 pregnant female mice were used for the hippocampal neuronal cultures (1 or 2 females for each neuronal culture). The animals were maintained under a 12 h light/dark cycle in type II cages in specific pathogen-free conditions (microbiological health status available). Animals were fed with regular rodents chow and tap water ad libitum. All animals used in this experiment were treated accordingly to Decreto-Lei 113/2013, present in the Portuguese legislation.

In order to obtain hippocampal cells cultures, we sacrificed wild-type pregnant C57BL/6 female mice at gestation day 16 by cervical displacement, performed by a qualified person to do so, in order to preserve molecular signaling in neurons. Embryos were removed from the female womb, and the intact brain was retrieved from the embryos and dissected to obtain the hippocampi. Hippocampi were isolated in Ca^{+}-free and Mg^{2+}-free Hank's balanced salt solution (HBSS) (maintains the equilibrium (e.g., pH and osmotic concentration) needed for neurons survival).

Neuronal cultures were prepared in a laminar flow cabinet. Hippocampi were dissociated with trypsin (0.25% in HBSS, for 10 minutes at 37ºC), washed in 10% fetal bovine serum diluted in HBSS (to stop trypsin activity), and washed in HBSS to remove serum (to prevent glia cells growth). Hippocampi were mechanically dissociated to separate individual cells from the connective tissue and maintained in serum free neurobasal medium, supplemented with B27 (1:50 dilution; Gibco), glutamate (25µM), glutamine (0.5mM), and gentamicin (0.12mg/mL) in poly-D-lysine (PDL) coated plates at a density of $9x10^4$ cells/cm^2 on 6 well plates or $2x10^5$ cells/cm^2 on coverslips. Cultures were kept at 37°C in a humidified incubator with 5% CO_2/95% air for 14 days in vitro (DIV). During the incubation period, cultures were regularly examined to make sure that cells were healthy and viable.

METH (100µM) was added to the neurons at 14DIV, and cultures incubated for additional 24 hours at 37ºC 5% CO_2/95% air. This METH dose was previously tested within a range of physiological doses in our laboratory following in vivo data from other authors (Fornai et al, 2004; Fornai et al, 2007), and we previously observed that, in our model, it does not affect neuronal death neither causes mitochondrial damage.

2.2. mRNA semi-quantification by real time PCR

At 15 DIV, total RNA was extracted from neurons, in order to assess and validate the presence of the genes with altered expression. The extraction was achieved using TRIzol reagent with PureLink® RNA mini kit (Ambion by life technologies), according to the manufacturers' instructions. Briefly, cells were scraped using 1mL of TRIzol® Reagent and incubated for 5 minutes. Chloroform (200µL) was added to the tube, shaken by hand for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 12,000 xg for 15 minutes at 4°C. The upper, colorless phase (≈ 400µL) containing the RNA was transferred to a fresh RNase-free tube, and an equal volume of 70% ethanol was added and samples were vortexed. Samples were then centrifuged at 12,000 xg for 15 seconds, at room temperature. Samples were washed 2 times for 15 seconds, at room temperature, and RNA was extracted in RNase-Free Water (30µL) by centrifugation at 12,000 xg for 2 minutes, at room temperature.

RNA concentration was assessed with NanoDrop 1000 (Thermo Scientific), and RNA quality and integrity with Experion Automated Electrophoresis System (BioRad). Samples showing RNA degradation or contamination by DNA were discarded. Samples were kept at -80ºC until further use.

RT-qPCR is performed in two steps: reverse transcription and real time PCR. This means that reverse transcription and real time PCR are done in different tubes, with different optimized buffers, reaction conditions and priming strategies (Basic Principles of RT-qPCR, Thermo Fisher Scientific).

cDNA synthesis was performed by reverse transcription using 1µg of the extracted RNA resorting to RT^2 HT First Strand Kit (Qiagen), following manufacturers' instructions. The obtained cDNA was stored at -20°C.

Binding of Sybr green (iQ™ SYBR® Green Supermix, Bio-Rad), a nucleic acid dye, to double stranded cDNA will emit fluorescence that will be used by the system to create a real time measurement of the amount of genetic material present. A higher amount of fluorescence detected means a higher amount of double-stranded cDNA. The lowest, but sufficient fluorescence detected will determine the threshold cycle (C_T), later used to quantify the initial RNA concentration.

To do so, we designed specific primers to the selected genes and to the reference genes (table I). Five genes (18s, Rpl19, Dcbld1, Rab7 and Ywhaz) were analysed to check their potential as reference genes. The chosen primers must hybridize with a specific region of the gene to avoid unspecific hybridization, and the amplicon (i.e., amplified region) should be between 75 to 200 base pairs long. For both amplicons and primers, the GC content should remain between 50% and 60%, regions with single-bases repeats should be avoided

and secondary structure formation ought to be avoided as well. In addition to these precautions, primers melting temperature should be maintained between 50°C and 65°C and G's and C's should be placed on ends of primers (Real-Time PCR Applications Guide, Bio-Rad). Primers were designed using *Beacon Designer™*.

Table I- Specific primers used on RT-qPCR .

Gene		Primers
Optn	Forward	5'GGGCAATGAAGGAGATGAAG 3'
	Reverse	5'TGGCTCACAGTCAGTTCT 3'
Olfm4	Forward	5'CTGCCAGTGTTCTGTTTC 3'
	Reverse	5'CTTCTCCATGACCTCTACTC 3'
Rasgrp1	Forward	5'GCAGAGGTCTTCATCAAG 3'
	Reverse	5'CAGCAGTTCAGTCATCTC 3'
RelB	Forward	5'GAAGTCCACCAACACATC 3'
	Reverse	5'CTGAACACCACGGATATG 3'
Ebf3	Forward	5'AGATTACGGCTTCCAGAG 3'
	Reverse	5'GGTTATTGTGAGGCATCC 3'
18s	Forward	5'AAATCAGTTATGGTTCCTTTGGTC 3'
	Reverse	5'GCTCTAGAATTACCACAGTTATCCAA 3'
Rpl19	Forward	5'TAGGGAAGAGGAAGGGTA 3'
	Reverse	5'AGGTACAGGCTGTGATAC 3'
Dcbld1	Forward	5'GTGACTGTCCTCTTCAAG 3'
	Reverse	5'CTCCTGCTATGTCTCTAC 3'
Rab7	Forward	5'CACAATAGGAGCGGACTT 3'
	Reverse	5'CACCAGAACACAGCAATC 3'
Ywhaz	Forward	5'GATGAAGCCATTGCTGAACTTG 3'
	Reverse	5'GTCTCCTTGGGTATCCGATGTC 3'

Il-1β	Forward	5'GCCCATCCTCTGTGACTCAT 3'
	Reverse	5'AGGCCACAGGTATTTTGTCG 3'
TNF	Forward	5'CTCACACTCAGATCATCTTC 3'
	Reverse	5'GAGAACCTGGGAGTAGATAAG 3'
iNOS	Forward	5'GTGGTGACAAGCACATTTGG 3'
	Reverse	5'AAGGCCAAACACAGCATACC 3'
Il-6	Forward	5'CACAAGTCCGGAGAGGAGAC 3'
	Reverse	5'CAGAATTGCGATTGCACAAC 3'
TGF-β	Forward	5'TGAGTGGCTGTCTTTTGACG 3'
	Reverse	5'GTTTGGGACTGATCCCATTG 3'

RT-qPCR was performed in METH-treated vs non-treated neurons in order to confirm the differential expression of the selected candidates. The samples were prepared accordingly to table II and the protocol used is described in table III. qPCR is executed in *iQ5 Multicolor Real-Time PCR Detection System* (Bio-Rad).

Table II- qPCR Reaction Setup (adapted from iQ™ SYBR® Green Supermix).

Component	Volume per 20µL reaction	Final Concentration
iQ™ SYBR® Green Supermix	10µL	1x
Forward and Reverse Primers	Variable	200nM
DNA Template	1µL	1µg
H_2O	Variable	-
Total reaction mix volume	20µL	-

Table III- qPCR Reaction Protocol.

	Temperature	Duration
Cycle 1 (1x)	94.0°C	03:00
Cycle 2 (40x)	---	---
***Step 1**	94.0°C	00:15
***Step 2**	60.0°C	00:20
Cycle 3 (81x)	55.0°C - 95.0°C Increase set point temperature after cycle 2 by 0,5 °C	00:30

cDNA quantification may be absolute or relative, calculated through various processes. In this case, quantification was relative, using both Livak and Pfaffl methods for efficiency correction. Relative quantification was performed by comparing the target gene expression to the reference gene expression, in both control and treated conditions. Livak method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other, while Pfaffl method assumes that each gene has the same amplification efficiency in treated and control samples, but it is not necessary that the target and reference genes have the same amplification efficiency (Real-Time PCR Applications Guide, Bio-Rad; figure 5). Results were normalized using one reference gene out of the five tested.

$$\text{Livak Method: } 2^{-\Delta\Delta CT}$$

$$\text{Pfaffl Method: } \text{Ratio} = \frac{(E_{target})^{\Delta Ct,\ target(calibrator-test)}}{(E_{ref})^{\Delta Ct,\ ref(calibrator-test)}}$$

Figure 5. Livak and Pfaffl quantification methods. Formulas used to quantify qPCR results.

2.3. Protein extraction and western blot analysis

Proteins being translated for the genes confirmed to have a differential expression in neurons treated with METH vs non-treated neurons were evaluated by western blot, using specific antibodies.

Protein extraction was performed at 15DIV. Neurons were washed with ice-cold PBS (twice) and lysed with RIPA (150mM NaCl, 50mM Tris–HCl, pH 7.4, 5mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5), supplemented with 50mM sodium fluoride (NaF), 1.5mM sodium orthovanadate (Na_3VO_4), 1mM dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1mM phenylmethylsulfonyl fluoride (PMSF), CLAP (1µg/ml

chymostatin, 1µg/ml leupeptin, 1µg/mL antipain, 1µg/mL pepstatin; Sigma). Protein extracts were kept at -80ºC until further use.

Protein was quantified using the Bicinchoninic acid (BCA) method, and samples were denaturated using 5x denaturating buffer (625mM Tris, pH 6.8, 10% SDS, 500mM DTT, 50% glycerol and 0.01% bromophenol blue), added to a 1x final concentration and boiled at 95ºC for 10 minutes.

Protein expression was analysed by Western Blot, using 40µg of sample, loaded into a 10% SDS-page gel, that runs at 60V (stacking gel) and 80V (resolving gel). The ladder used was PageRuler™ Plus Prestained Protein (Thermo Fisher Scientific). Proteins were transferred into a PVDF membrane using Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes incubated with primary antibody overnight, at 4°C (anti-Olfm4 1:1000, D6Y5A, kindly provided by Cell Signaling; anti-GAPDH 1:100000, 5G4 HyTest; anti-Laminin 1:1000, ab7463 Abcam; anti-β1 Integrin 1:3000, ab52971-100 Abcam; anti-ZO-1 2µg/mL, 40-2200 Invitrogen; anti-Claudin-5 1:1000, ab53765 Abcam; anti-pSrc 1:1000, #6943S Cell Signaling; anti-Src 1:5000, ab109381 Abcam), and with secondary antibody for 1 hour, at room temperature (HRC conjugated anti-rabbit 1:10000, A0545 Sigma-Aldrich; HRC conjugated anti-mouse 1:3000, 31432 Thermo Fisher Scientific). Membrane revelation resorted to Clarity™ Western ECL Substrate (Bio-Rad) and to ChemiDoc (ChemiDoc™ MP System, Bio-Rad). Results were quantified and processed with ImageLab software (Image Lab™ Software, Bio-Rad).

2.4. Immunocytochemistry

Protein expression in cultured hippocampal neurons was also analysed by immunocytochemistry. At 15DIV, neurons cultured in coverslips were fixed with 4% paraformaldehyde and 4% sucrose in PBS, permeabilized with 0,25%Triton X-100 in PBS for 10 minutes at room temperature, and blocked with 5% BSA in PBS with 0,1%Tween-20 for 1 hour at room temperature. Neurons incubated with primary antibody (anti-Olfm4 1:50, D6Y5A, kindly provided by Cell Signaling; anti-ZO-11:100, 40-2200 Invitrogen; anti-claudin-5 1:100, ab53765 Abcam; anti-pSrc 1:100, #6943S Cell Signaling; anti-β3 tubulin 1:1000, G7121 Promega; anti-GFP 1:500, 11814460001 ROCHE Sigma-Aldrich) diluted in 0.5% BSA in PBS-0,1%Tween-20 overnight, at 4°C. Secondary antibodies (Alexa 594 anti-rabbit 1:500, A11012 Life Technologies; Alexa 488 anti-mouse 1:500, A11001 Life Technologies; Alexa 488 anti-rabbit 1:500, #4412S Cell Signaling; Alexa 568 anti-mouse 1:500, A11004 Life Technologies) were incubated for 1 hour, at room temperature. Hoechst 33342 (0.5µg/mL; B2261 Sigma-Aldrich) was used as a nuclear marker, and incubated with neurons for 10 minutes, at room temperature. Coverslips were mounted in Fluorescence Mounting Medium

(DAKO) and imaging was performed using Leica DMI 6000 Widefield microscope. Images were quantified using image analysis software FIJI.

2.5. Zymography to assess MMP2 and MMP9 activity

MMP2 and MMP9 activity was evaluated using a gelatin zymography assay. Briefly, 30µg of protein was mixed with sample buffer (10% SDS, 120mM sucrose, 0.25M Tris pH6.8); 1µL of buffer per 2µL of sample. Samples were loaded into a gelatin acrylamide gel (resolving gel: 10% polyacrylamid gel with 0.1% gelatin as a substrate; stacking gel: 5% polyacrylamide gel) that runs at 80V, at room temperature. After running, SDS was removed from the gel with a washing solution (2% Triton X-100) and incubated, overnight, at 37°C, with MMP substrate buffer (40mM Tris, 10mM $CaCl_2.(2H_2O)$, 3mM NaN_3 in ultrapure H_2O until 1000mL, pH7.5). Gel was then stained with Coomassie blue solution (1.2mM in a 10% acetic acid and 40% methanol solution) for 20 minutes and destained (20% methanol, 10% acetic acid, in distilled H_2O) until bands were visible.

2.6. FRET assay

FRET was performed to detect c-Src activity in response to METH exposure as well as calcium accumulation in endoplasmic reticulum and RhoA activation. This technique relies on probes with two kinds of fluorophores: the donor and the acceptor. When excited with the adequate light, donor emits energy that excites the acceptor fluorophore which then emits detectable fluorescence. This only occurs when donor and acceptor fluorophores are in close proximity (figure 6).

In order to do so, we plated neuronal cells ($9x10^4$ cells/cm^2) on culture dishes (µ-Dish 35mm, iBidi) that were transfected (as detailed below) with FRET probes for c-Src activation (K Ras Src YPet; Ouyang et al, 2008), for calcium present in the endoplasmic reticulum (D1ER; Palmer et al, 2004) or RhoA activation (Raichu-RhoA; Yoshizaki et al, 2003).

Images were obtained with Leica DMI6000B Widefield inverted microscope with a mercury metal halide bulb as excitation light source, integrated with EL6000 light attenuator. High-speed low-vibration external excitation/emission filter wheels (equipped with CFP/YFP excitation and emission filters) were mounted on the microscope (Fast Filter Wheels, Leica Microsystems) and a 440–520 nm dichroic mirror (CG1, Leica Microsystems) and a PlanApo 63x1.3NA glycerol immersion objective were used for acquiring donor (CFP) and acceptor (FRET) images. Images were acquired using 2x2 binning and a digital CMOS camera (ORCA-Flash4.0 version 2, Hamamatsu Photonics). At each time point, donor and FRET images were sequentially acquired using different filter combinations (donor excitation plus donor emission, and donor excitation plus FRET emission, respectively).

Obtained images were analysed resorting to FIJI image analysis software. Background was subtracted from all slices, in both channels, using a Kalman filter implementation. Segmentation was achieved on a pixel-by-pixel basis using the local Phansalkar algorithm. After background subtraction and filtering, ratiometric images (Donor/FRET for c-Src probe, FRET/Donor for the ER calcium release probe or FRET/Donor for RhoA probe) were generated in intensity-modulated display mode using the FRET images as intensity modulators in FIJI (Socodato et al, 2015).

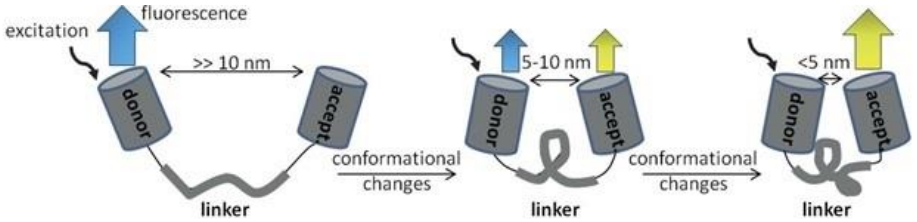


Figure 6. FRET assay. Schematic explaining FRET mechanism. When excited, donor emits energy that, when in proximity, excites the acceptor fluorophore which then emits detectable fluorescence (adapted from Lindquist and Niesner, 2015).

2.7. Gene expression modulation

The vector of interest (shRNA.Olfm4) was acquired from VectorBuilder (Cyagen) (target sequence: AGGAGTATGTCCAGCTAATAA). We used, as a control, DsRed vector in infected neurons or Venus vector in transfected neurons.

To produce the plasmid DNA, bacteria were plated in AGAR-ampicillin (100µg/mL) plates and incubated overnight, at 37°C. After the incubation period, one single colony was collected and pre-inoculated in LB-medium (5mL), supplemented with ampicillin, that incubates at 37°C, with agitation, for approximately 12 hours. Bacteria were then diluted in 150mL of LB-medium supplemented with ampicillin, which incubates overnight, at 37°C, with agitation. Afterwards, the medium was centrifuged in order to pellet bacteria. NucleoBond® Xtra Midi/Maxi kit (Macherey-Nagel) was used to purify the plasmid DNA, following manufactures' instructions.

Lentiviruses production was performed in HEK293 cell line transfected with jetPRIME® (Polyplus-transfection), following manufactures' instructions. Plasmid DNA (10µg) was diluted in jetPRIME® buffer and vortexed for 10 seconds. jetPrime® reagent was added and vortexed for 10 seconds. After incubating at room temperature, for 10 minutes, the transfection mix was added to the cells After 24 hours, the medium was collected, centrifuged at 3000rpm for 15 minutes, at 4°C, and the supernatant was recovered and aliquoted and stored at -80°C.

2.7.1. Neuronal infection with lentivirus

For the knockdown gene expression experiments, cultured hippocampal neurons were infected at 11 DIV. For that, ¼ of the viral suspension diluted in culture conditioned medium was added to the neurons and, after 24 hours, this medium was replaced by culture conditioned medium. At 14 DIV, neurons were treated with METH 100µM for 24 hours, and protein was extracted for western blot analysis as described above.

2.7.2. Neuronal transfection

To knock-down gene expression in neurons we also used transfection by calcium phosphate co-precipitation method at 12 DIV.

To do so, 100µL of precipitate (enough for two 10mm coverslips) was prepared by diluting 4µg of plasmid DNA in 1x TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, pH 7.3) in a final volume of 45 µL. 5 µL of 2.5M $CaCl_2$ (prepared in 10mM HEPES) was added dropwise to the TE/DNA mixture (final concentration of 250 mM); the mix was gently vortexed. The DNA/ TE/ $CaCl_2$ solution was added, dropwise, to 50µL of 2x HEBS solution (0.4mM NaCl, 10mM KCl, 1.4mM Na_2HPO_4, 11mM Dextrose, 84mM HEPES, pH 7.2). The precipitate was then developed at room temperature for 30 minutes, protected from light, and vortexed every 5 minutes. In the meantime, each coverslip was placed in a well containing 200µL of conditioned medium and 50µL of 10mM kynurenic acid, to a final concentration of 2 mM, and cells were returned to the 37ºC/5% CO_2 incubator. When ready, precipitate (50µL) was added, dropwise, to the each coverslip, and cells were incubated for, approximately, 3 hours. Then, neurons were incubated with 1mL of HCl-acidified 2 mM kynurenic acid and cells returned to the incubator for 20 minutes. Finally, coverslips were transferred to the original wells, containing their conditioned medium. At 14 DIV, cells were exposed to METH (100µM) for 24 hours, and neurons were fixed as described above.

The same procedure was used to express Venus (i.e., control vector) and a constitutively active form of RhoA (RhoA CA).

2.8. Morphological Analysis

To evaluate neuronal morphology, we assessed neurite length and branching as morphological characteristics. Neurite length was assessed using FIJI (Schindelin et al, 2012) software and the plugin NeuronJ (Meijering et al, 2004) by tracing neurites in neurons transfected with either Venus or RhoA CA, in control and exposed to METH neurons. The obtained data was analysed for neurite length and was used to evaluate neuronal branching by Sholl Analysis. For this, we resorted to MATLAB (MATLAB 6.1, The MathWorks Inc.,

Natick, MA, 2000) software, Bonfire Program (Langhammer et al, 2010) and NeuronStudio (Wearne et al, 2005) that allow us to draw concentric circles around the cell body (6 μm apart) and determine the number of intersections between the circles and neurites.

2.9. Statistical Analysis

The results obtained in each experiment were normalized by the control mean. Data is presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using GraphPad Prism7, using Student's t test or One-way analysis of variance (ANOVA), followed by adequate post-tests.

Chapter 3: Results

3.1. Selection of Candidates

The data supporting this work was obtained in previous experiments, performed in our laboratory, involving an RNA sequencing approach to assess genes showing altered expression in cultured hippocampal neurons, in response to METH exposure. RNA was extracted from soma and neurite fractions of hippocampal neuronal cultures exposed, or not, to METH (Pertz et al, 2008). RNA sequencing, as the name points, is the transcriptome (i.e., all transcribed RNA) profiling through deep-sequencing technologies. It provides, for example, information about gene transcriptional structure and expression levels of transcripts during development and under different conditions (Wang et al, 2009). In this particular research, it granted valuable information on how METH might be altering gene expression.

Because METH was previously seen to affect the morphology of hippocampal neurons, and taking into consideration that it is now accepted that changes in neuronal morphology are strongly associated with the addictive behaviors, the hippocampus was the brain region selected for this study (Thompson et al, 2004). Beyond this, it is also known that neurodegeneration associated to METH-use is noticeable in brain regions with notable IgG immunoreactivity (i.e., hippocampus and amygdala) and during hyperthermic conditions (mouse body temperatures >40.5 °C). It was described that the effect of both BBB disruption and hippocampal damage might be sufficient to compromise cognitive function (Bowyer and Ali, 2006). Besides, the hippocampus is described as being part of the reward mechanism, since it is involved in long-term episodic memory, contributing to reward-related behaviors (e.g. reinforcement learning or reward-guided motivation) (Davachi, 2006; Davidow et al, 2016; Squire et al, 2004).

The obtained results were first analysed through a bioinformatics approach in order to identify the genes differentially expressed in neurons exposed to METH vs non-exposed neurons, in the soma fraction. This analysis was outsourced to the Brabaham Bioinformatics Centre (Cambridge, UK). From this analysis we have identified 128 genes with altered expression when exposed to METH. Each of these genes was thoroughly analysed recurring to information available in multiple sources, from scientific articles to databases *(Genecards*, *NCBI* and *Uniprot*), and classified according to four categories, based on their function: metabolism, cell signaling, cell cycle or transcription/transduction. From this analysis we have selected 5 genes that were likely to be related to neuroinflammation and neuron/glia interplay: Optn, Olfm4, Rasgrp1, RelB and Ebf3. These genes showed a differential expression in the soma of METH treated vs non-treated hippocampal neurons: the first two genes were upregulated, and the last three were downregulated in the soma of neurons exposed to METH (table IV). Besides the function of these selected genes, we also took into

consideration how altered the mRNA expression was, how the selected proteins affect the cerebral function and how it would be relevant towards METH addiction. Since the selected genes have either a direct or indirect link to inflammatory mechanisms, they might also alter neuroinflammatory pathways and physiological responses.

The Rasgrp1 gene translates into the RAS Guanyl Releasing protein (90 kDa), that is responsible for activating Ras proteins, through switching bound GDP for GTP. Overexpression of this protein results in increased activation of Ras proteins. Rasgrp1 is also responsible for activating the Erk/MAP kinase pathway and regulating the development, homeostasis and differentiation of T and B cells. (Hartzell et al, 2013; RASGRP1. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=RASGRP1&keywords=rasgrp1).

The RelB gene encodes the RelB Proto-Oncogene protein (62 kDa), a NF-κB subunit. It belongs to the NF-κB transcription factor family and it can both activate and repress the NF-kB complex, through interactions with RelA. It controls dendritic cells maturation and may be used as a therapeutic target to manipulate T cells response (Marienfeld et al, 2003; Shih et al, 2012).

The Ebf3 gene encodes the Early B-Cell Factor 3 (65 kDa) which belongs to the EBF transcription factors family and is involved on B cells differentiation, bone development and neurogenesis. It has been suggested that this protein might act as a tumor suppressor, since its expression is silenced in brain tumors. Microglial cells activation, usually occurring in neuroinflammation and neurodegenerative diseases, leads to a decrease on Ebf3 expression on mice hippocampus (Lee et al, 2013; Zhao et al, 2006; EBF3. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=EBF3).

The Optn gene encodes for Optineurin (66 kDa), which is, composed of multiple coiled-coil domains, an ubiquitin-binding domain and a C-terminal zinc finger. This protein is involved in Golgi complex maintenance, membrane trafficking and exocytosis. It has been identified as a negative regulator of NF-kB signaling and it was hypothesized that it uses TNF or Fas-ligand pathways to modulate apoptosis, inflammation or vasoconstriction. It was also described that optineurin is overexpressed in response to viral infections (Nakamura et al, 2014; Sudhakar et al, 2009; OPTN. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=OPTN; Q96CV9 (OPTN_HUMAN). Available from: http://www.uniprot.org/uniprot/Q96CV9#section_comments).

The Olfm4 gene translates into Olfactomedin4 protein (57 kDa), a matrix glycoprotein that belongs to the olfactomedin domain-containing protein family. It has been described as an anti-apoptotic factor, being preferably expressed in non-neuronal tissues, like the gastrointestinal tract and bone marrow. Olfm4 expression is known to be regulated by granulocyte colony-stimulating factor (G-CSF), transcription factor PU.1 (PU.1) and NF-κB, and has been linked to inflammatory processes, since deleting this gene increases immune

defense against *Staphylococcus aureus* (Liu et al, 2010; Liu et al, 2013; Tomarev and Nakaya, 2009; Zhang et al, 2002). It has been pointed that Olfm4 interacts with matrix metalloproteinases (MMP9) and integrins, and that it is also involved in cell adhesion and tumor metastasis (Park KS et al, 2012).

Table IV- mRNA expression in response to METH exposure. Log2FoldChange reveals if METH causes an increase or a decrease in gene expression.

Gene	Base Mean	log2FoldChange*	*P* value*
Rasgrp1	998,487	-0,371	0,045
RelB	341,187	-0,538	0,031
Ebf3	88,840	-0,799	0,020
Optn	1286,724	0,296	0,041
Olfm4	213,093	0,610	0,018

*Results extracted from the RNAseq analysis outsourced to Brabaham Bioinformatics Centre (Cambridge, UK)

3.2. mRNA expression levels evaluated through RT-qPCR

In order to validate the data obtained in our RNAseq analysis, and evaluate the mRNA expression levels of the selected candidates (Optn, Olfm4, Rasgrp1, RelB and Ebf3), cultured hippocampal neurons were treated, or not, with METH for 24 hours, and total RNA was extracted. mRNA levels were assessed by reverse transcription quantitative PCR and quantification was performed using the expression of a reference gene. The identification of the appropriate reference gene is of crucial relevance to this step, since it is an internal reaction control whose expression levels are unaffected by experimental factors (reviewed in Kozera and Rapacz, 2013). For that, we tested several genes to assess if they could be used as reference genes. The genes Rpl19, Dcbld1 and Rab7 were selected from the RNAseq analysis, as genes whose expression levels were not differentially affected in neurons exposed or not to METH. Moreover, we also used Ywhaz (Tyrosine 3-Monooxygenase; belongs to the 14-3-3 family of proteins that mediate signal transduction by binding to phosphoserine-containing proteins and regulates several general and specialized signaling pathways; Ywhaz. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=YWHAZ), a gene that was previously described in our lab as a gene whose expression is not altered by METH administration, and 18S, a gene widely used as a reference gene. We end up selecting the Ywhaz gene, since this was the only that showed

no significant difference (i.e. a difference equal or smaller than 1 amplification cycle in qPCR) between neurons treated or not with METH. We quantified gene expression using two quantification methods (Livak and Pfalff) (Real-Time PCR Applications Guide, Bio-Rad).

The results previously obtained in the RNA sequencing, as referred in section 3.1 (see Table I) showed that Ebf3, Rasgrp1 and RelB had a decreased expression in the soma of neurons exposed to methamphetamine versus non-exposed neurons, while Optn and Olfm4 presented an increased expression. Using RT-qPCR, we observed that the gene expression levels of Rasgrp1, RelB and Ebf3 were not significantly altered in neurons treated with METH versus non-treated neurons, which may relate to the fact that we evaluated mRNA extracted from the entire neuron, while the RNAseq analysis only evaluated soma. We could not evaluate the expression of the Optn gene, since the amplification was not successful (figure 7).

As displayed in Table I, Olfm4 mRNA expression was seen to be significantly increased in hippocampal neurons exposed to methamphetamine comparing to non-exposed neurons in our RNAseq analysis. As represented in figure 8 we confirmed that upon exposure to METH the Olfm4 mRNA expression levels were significantly increased in primary cultures of hippocampal neurons (figure 8, $p<0.05$ as obtained through unpaired t-testing). Based on these results we further investigated the putative role of Olfm4 under exposure to psychostimulants.

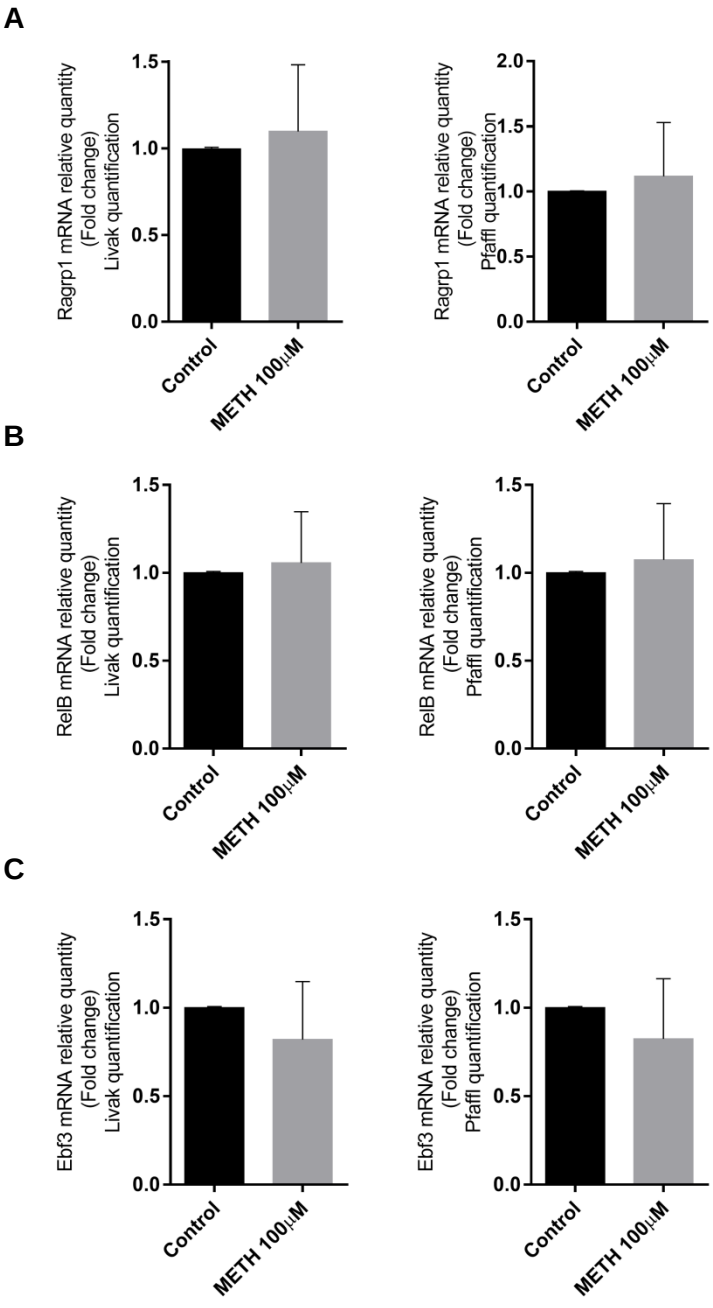


Figure 7. qPCR results for Rasgrp1 (A), RelB (B) and Ebf3 (C) genes. No differences could be observed for the gene expression levels of Rasgrp1 (A), RelB (B) and Ebf3 **(**C) when comparing mRNA obtained from hippocampal neurons exposed to methamphetamine with that of control cultures.. Quantification was executed resorting to Livak and Pfaffl methods. The results are presented as the mean ±SEM of 3 (A and C) or 5 (B) independent experiments. Statistical analysis was performed using an unpaired t-test.

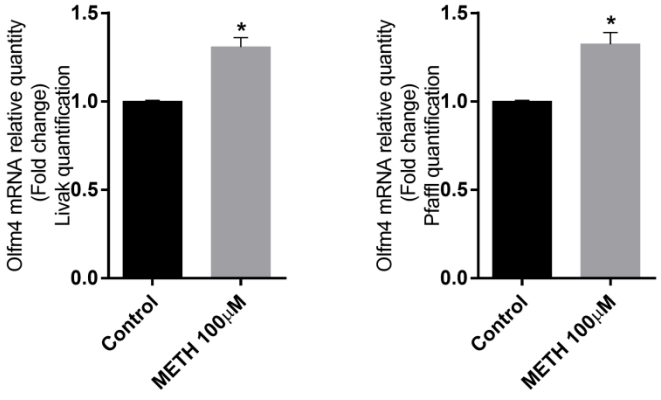
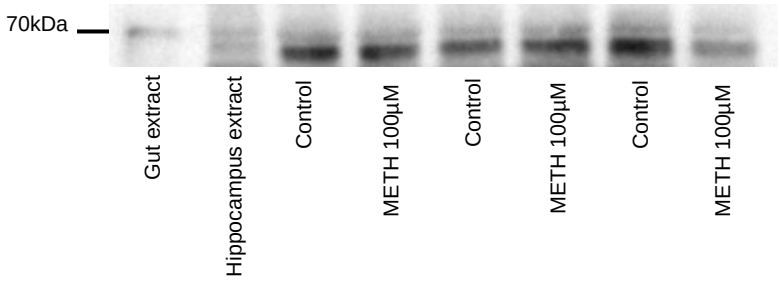


Figure 8. Olfm4 mRNA increased expression levels under methamphetamine exposure. We observed a significant increase for the gene expression levels of Olfm4 when comparing mRNA obtained from hippocampal neurons exposed to methamphetamine with that of control cultures. Quantification was executed resorting to Livak and Pfaffl methods. The results are presented as the mean ±SEM of 3 independent experiments. Statistical analysis was performed using an unpaired t-test. The results are the average of ±SEM of 3 independent experiments performed in independent experiments. Statistical analysis was performed using an unpaired t-test with Welch's correction. *p<0.05

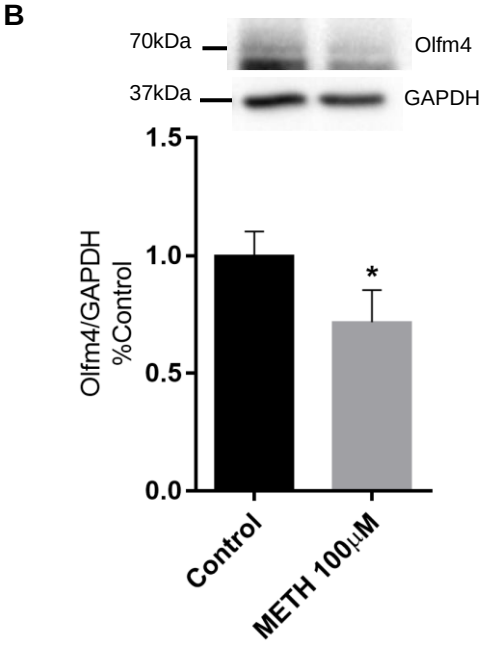
3.3. Olfm4 expression levels in hippocampal neuronal cultures

Next, we evaluated the effect of METH in the protein expression levels of Olfm4. In order to do so, we produced protein extracts from cultured hippocampal neurons exposed or not to METH 100 μM for 24 hours, and analysed them by western blot. Of note, Olfm4 protein levels were decreased in response to METH exposure (figure 9B, $p<0.05$), which was not in accordance with the expected results considering that the mRNA expression levels of Olfm4 were increased both in the RNAseq analysis and the qPCR. Mouse gut extract was used as a positive control, since Olfm4 is highly expressed in this tissue (Tomarev and Nakaya, 2009; Zhang et al, 2002) (figure 9A).

A


70kDa
Gut extract
Hippocampus extract
Control
METH 100µM
Control
METH 100µM
Control
METH 100µM

B


70kDa
Olfm4
37kDa
GAPDH
Olfm4/GAPDH
%Control
1.5
1.0
0.5
0.0
*
Control
METH 100µM

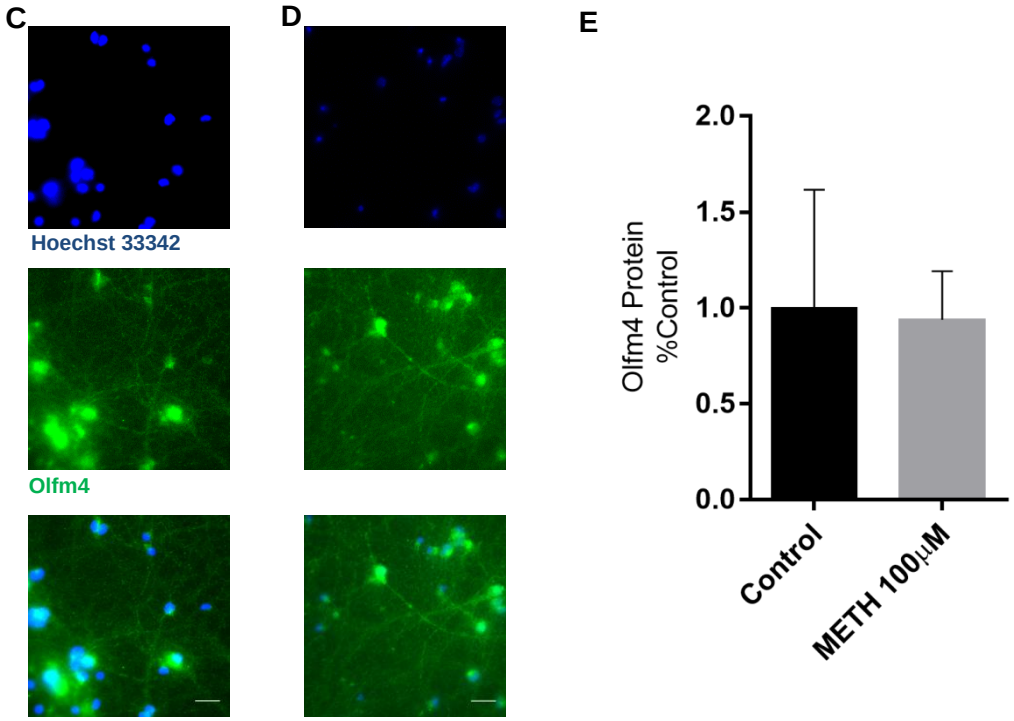


Figure 9. Olfm4 protein expression in hippocampal neurons. (A) Evaluation of Olmf4 protein levels in gut extract and hippocampal extract used as positive controls, and in neuronal cultures. (B) Protein extracts from hippocampal neuronal cultures analysed by western blot using an antibody for Olfm4, weight is defined as 70kDa. Representative images of control (C) vs Methamphetamine (100μM) (D) 15 DIV hippocampal neuronal cultures showing an immunocytochemistry using an antibody against Olfm4 (green) and the nuclear marker Hoescht (blue). Images were obtained using a Leica DMI 6000 Widefield microscope and quantified (D) using image analysis software FIJI. Columns represent mean ±SEM for 3 independent experiments performed in different preparations and were analysed using a paired t-test. *p<0.05

3.4. Finding a putative role for Olfm4 in hippocampal neurons

The primary goal of this work was to assess if METH administration to the cultured hippocampal neurons would affect the expression of genes that could be involved in the modulation of inflammation. The first issue addressed was if we could detect mRNA levels by RT-qPCR of a series of elements usually associated with inflammation in neurons: interleukin (IL)-1β, TNF, inducible nitric oxide synthase (iNOS), IL-6 and tumor growth factor (TGF)-β. IL-1β is a pro-inflammatory cytokine, which has been described as a vital regulator of acute central nervous system inflammation. Expressing IL-1β is sufficient to cause a continued neuroinflammatory response, within the mouse hippocampus (Ramesh et al, 2013; Shaftel et al, 2007). TNF is a pro-inflammatory cytokine involved in starting and sustaining inflammatory responses (Belarbi et al, 2012). iNOS is expressed in neurons in response to a complex signaling cascade, causing iNOS-dependent nitric oxide generation, neuronal

dysfunction and eventually death (Heneka and Feinstein, 2001; Olivenza et al, 2000). IL-6 is also a pro-inflammatory cytokine produced by neurons is response to injury-related stimuli and is elevated in inflammatory states (Murphy et al, 1995). It is described as a pleiotropic cytokine since it is involved in several biological functions, such as immune regulation, hematopoiesis and oncogenesis (Kishimoto, 2010). Finally, TGF-β, an anti-inflammatory cytokine, regulates proliferation, differentiation, adhesion, migration, among other cellular processes. It is believed that it might contribute to diminish microglia activity in particular circumstances (reviewed in Biber et al, 2007; Brionne et al, 2003; Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=TGFB1).

Interestingly, although METH is described to induce neuroinflammation and neuronal cells are said to express the inflammatory elements above mentioned, we could not successfully amplify none of the genes coding for such elements, when using mRNA collected from hippocampal cultures exposed to METH and respective controls, which might indicate that neurons do not produce such factors in direct response to METH. For that reason, we could not further explore the possible relation of Olfm4 with the expression of these proteins/cytokines.

Olfm4 is, as already mentioned, a matrix glycoprotein. Several studies indicate that this protein is involved not only in neuroinflammatory processes, but also in extracellular matrix remodeling. Therefore, we decided to evaluate how METH was affecting relevant matrix proteins in our hippocampal cultures. It has been described that Olfm4 interacts with integrins (Parks et al, 2012), lectins and cadherins (Tomarev and Nakaya, 2009). With this in mind, we evaluated the effect of METH treatment (100 µM for 24 hours) in the expression levels of β1 Integrin (ITGB1), Laminin, Claudin5 (Cldn5) and Zonula occludens-1 (ZO-1), by western blot analysis. For both β1 Integrin and Laminin no detectable bands were present at the expected molecular weight when using adequate antibodies. For the expression levels of Cldn5 (Fig 10A) and ZO-1 (Fig 10B) no significant differences were observed in neurons treated with METH versus non-treated neurons. Of note, our laboratory recently reported that exposure to METH leads to translocation of Cldn5 from the membrane to the cytoplasm in an endothelial cell line, which was accompanied by a significant reduction of total Cldn5 expression (Fernandes et al, 2016), although this does not seem to be the case in our hippocampal cultures, we will further address this issue in chapter 3.5.

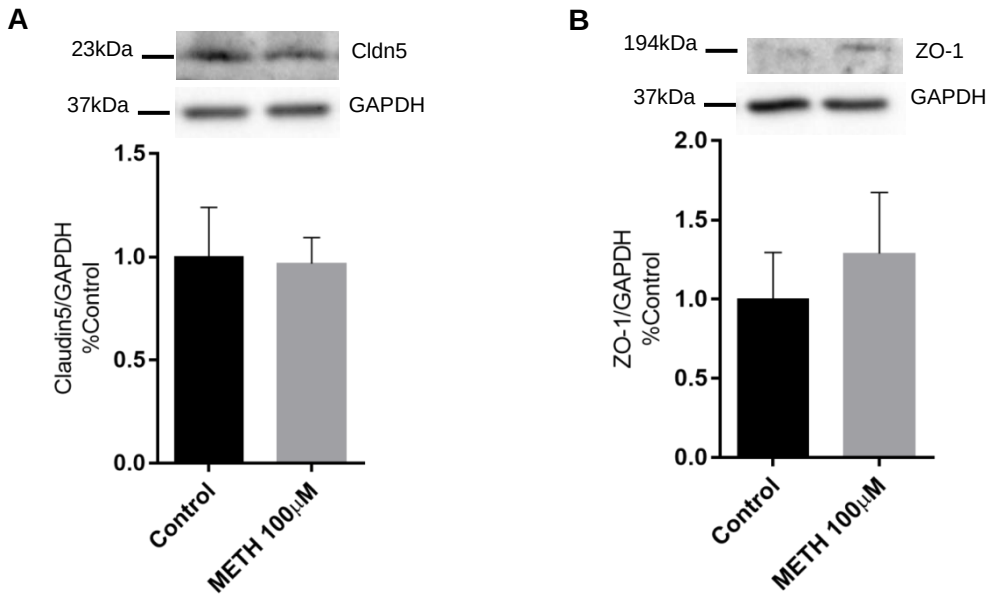


Figure 10. Claudin5 and ZO-1 protein levels in hippocampal neuronal cultures. Methamphetamine (100 μM) does not significantly affect the expression of either Claudin5 (A) or ZO-1 (B) in primary hippocampal cultures when compared to control conditions. GAPDH was used as a loading control. Columns represent mean ±SEM 3 independent experiments performed in independent experiments and were analysed using a paired t-test. *p<0.05

In endothelial cells, our laboratory has also reported METH-exposure increases the expression of MMP9 by increasing the expression of ILK (Integrin Linked Kinase) affecting cytoskeletal integrity (Fernandes et al, 2016). MMPs are zinc-containing endopeptidases, that, when activated, participate in the regulation of diverse physiological and pathological processes like the degradation of extracellular matrix, remodeling of tissues or shedding of cell surface receptors. MMPs are also involved in brain development, affecting several neurophysiological functions, like synaptic plasticity and long-term potentiation (reviewed in Brkic et al, 2015).

Src family proteins are involved in modulating MMPs (in particularly MMP2) activation through the ERK/Sp1 pathway (Kuo et al, 2006). More specifically, increased Src activation enhances the binding of Sp1 transcription factor leading to gene transcription (Kuo et al, 2006). MMPs are proteolytic zinc-containing endopeptidases that degrade ECM proteins. They are secreted into the extracellular milieu or
expressed in the plasma membrane (reviewed in Mulholland et al, 2016).

Bearing this in mind, we decided to determine if Src activation (i.e. Src phosphorylation, pSrc) was affected in hippocampal neurons by METH exposure. To do so, total Src and pSrc protein expression levels were analysed by western blot and results were

normalized for total Src levels. As represented in figure 11, at the tested level, we could not observe a significant difference between control and METH exposed conditions.

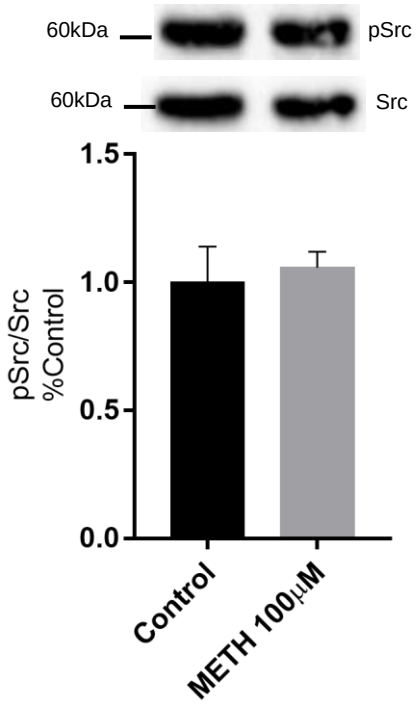


Figure 11. pSrc expression in METH exposed hippocampal cultures. Total amount of pSrc vs total Src determined by Western blot. Data represent mean ±SEM for 3 independent experiments performed in different preparations. Statistical analysis was performed using a paired t-test.

As the western blot results reflect a 24h time point after the exposure to METH, we further assessed Src activity under METH, conducting a FRET analysis in hippocampal neurons, to evaluate c-Src activation in response to METH exposure in real time (figure 12). As represented in figure 12A, c-Src activity increases in response to METH exposure, displaying a late-start (10 min delay) but steady and prolonged effect. Because calcium influx has been previously linked to Src activation (Rusanescu et al, 1995), we also conducted a FRET assay to measure the amount of calcium release by the endoplasmic reticulum (ER) (figure 12B). We observed that approximately 5 min after METH administration, the calcium present in the ER starts decreasing up to 20 minutes. At 20 minutes after METH administration, calcium levels rise and start decreasing shortly after, in a way that is compatible with a cycling function. Importantly, the augment in calcium release seems to correlate with the augmented c-Src activity observed.

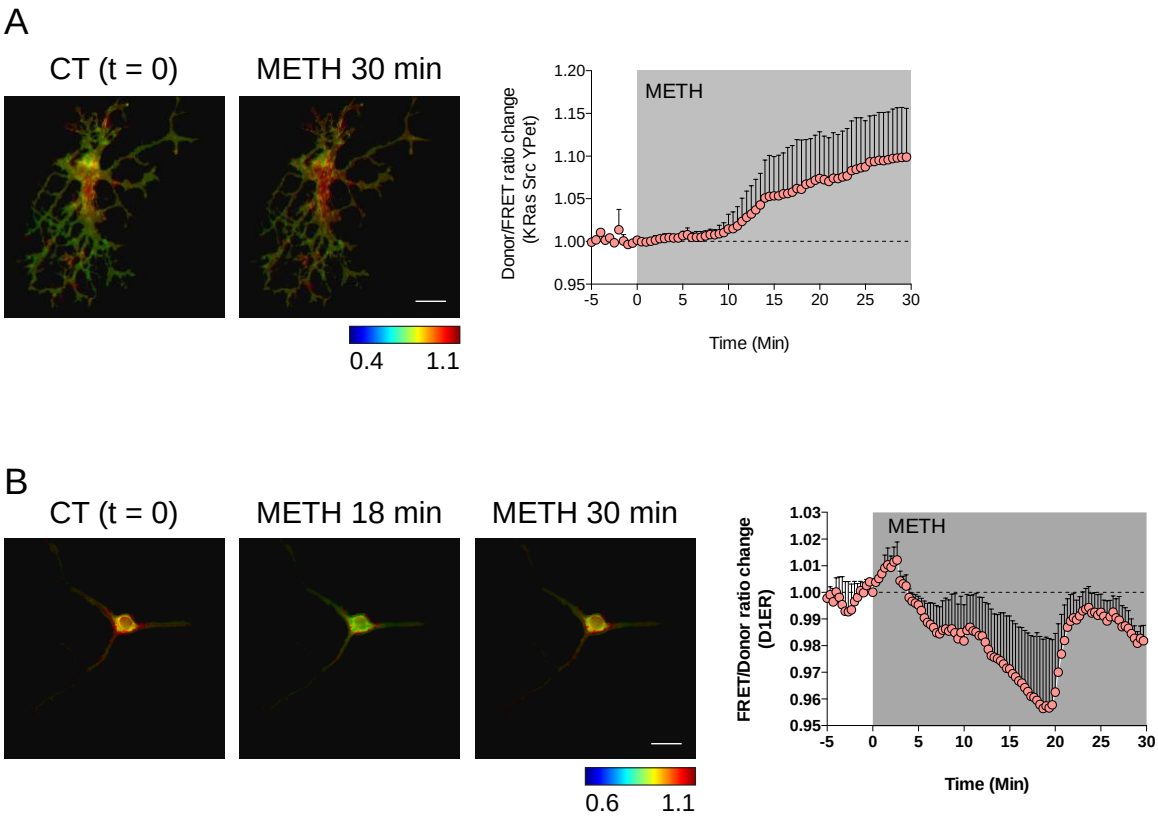


Figure 12. FRET analysis for c-Src activity and release of Calcium from the endoplasmic reticulum. (A) Primary hippocampal neurons expressing a c-Src FRET probe (K Ras Src YPet) were challenged with 100μM METH. Donor/FRET emission ratios before (control; t=0 in the images) and after METH administration were normalized at 0 min. Symbols represent the mean + SEM of two different and independent cells. The images show time-lapse (0 and 30 min) Donor/FRET ratios (min and max) coded according to the indicated pseudocolor ramp. Calibration bar 20 μm.
(B) Primary hippocampal neurons expressing an ER Calcium FRET probe (D1ER) were challenged with 100μM METH. FRET/ Donor emission ratios before (control; t=0 in the images) and after METH administration were normalized at 0 min. Symbols represent the mean + SEM of four different and independent cells. The images show time-lapse (0, 18 and 30 min) FRET/Donor ratios (min and max) coded according to the indicated pseudocolor ramp. Calibration bar 20 μm.

Arthur et al (2000) have suggested that Src activation leads to a decreased RhoA activation through p190RhoGAP activation. RhoA is a low-molecular-weight guanine nucleotide binding proteins that gets active by switching from an inactive GDP-bound state to an active GTP-bound state (Govek et al, 2005). RhoA is responsible for regulating stress fiber formation and cell contraction as well as for modulating neuronal morphology (reviewed in Gonzalez-Billault et al, 2012; Nobes and Hall, 1995; Ridley and Hall, 1992). RhoA is also involved in numerous cell functions like migration, adhesion, survival, cell division, gene expression and vesicle trafficking (Jaffe and Hall, 2005). The link between c-Src activation and RhoA modulation is of primary interest for us since we have also observed that METH leads to down activation of RhoA in our primary hippocampal culture. As shown in figure 13,

we used a FRET probe to assess RhoA activation state and observed a decrease in RhoA activation after 100 µM METH administration to the neurons. Although this link needs to be further explored, we can predict that the increased c-Src activation may lead to decreased RhoA activity, probably through p190RhoGAP. This decrease in RhoA activation may regulate METH-induced changes in neuronal morphology, which was observed by others and in previous experiments in our laboratory (Jedynak et al, 2007; Kiyatkin and Sharma, 2009; Marshall et al, 2007).

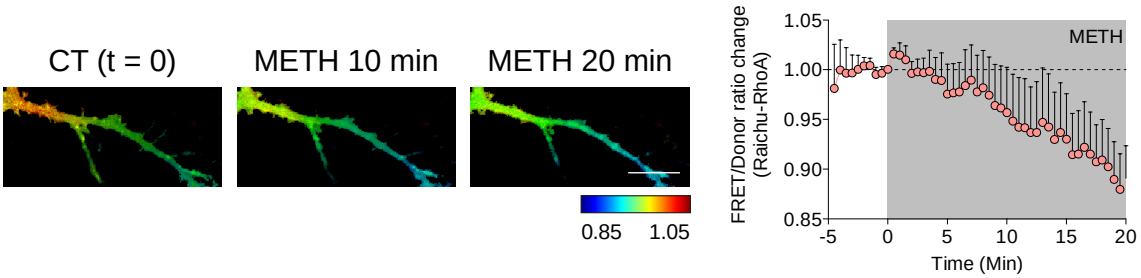


Figure 13. FRET analysis for RhoA activity. Primary hippocampal neurons expressing a RhoA FRET probe (Raichu-RhoA) were challenged with 100µM METH. Donor/FRET emission ratios before (control; t=0 in the images) and after METH administration were normalized at 0 min. Symbols represent the mean + SEM of 23 processes in five different and independent cells. The images show time-lapse (0, 10 and 20 min) Donor/FRET ratios (min and max) coded according to the indicated pseudocolor ramp. Calibration bar 5 µm.

RhoA, as mentioned above, is involved in modulating neuronal morphology. In order to assess the possible influence of RhoA in neuronal morphology when exposure to METH occurs, we evaluated neuronal length and branching in neurons transfected with Venus or a constitutively active form of RhoA. The obtained results show that methamphetamine induces a significant increase in neuronal length (figure 14A). However, when RhoA is constitutively expressed, we no longer see an effect of methamphetamine (figure 14B). Moreover, Sholl analysis demonstrates that METH exposure results in increased neuronal branching, especially closer to the cell body. RhoA expression leads to an overall decrease of branching and METH no longer appears to induce this sort of increase (figure 14C). These results appear to indicate that RhoA does have a role in neuronal morphology and that the decrease in RhoA activity in response to methamphetamine exposure is necessary for the morphological alterations prompted by METH.

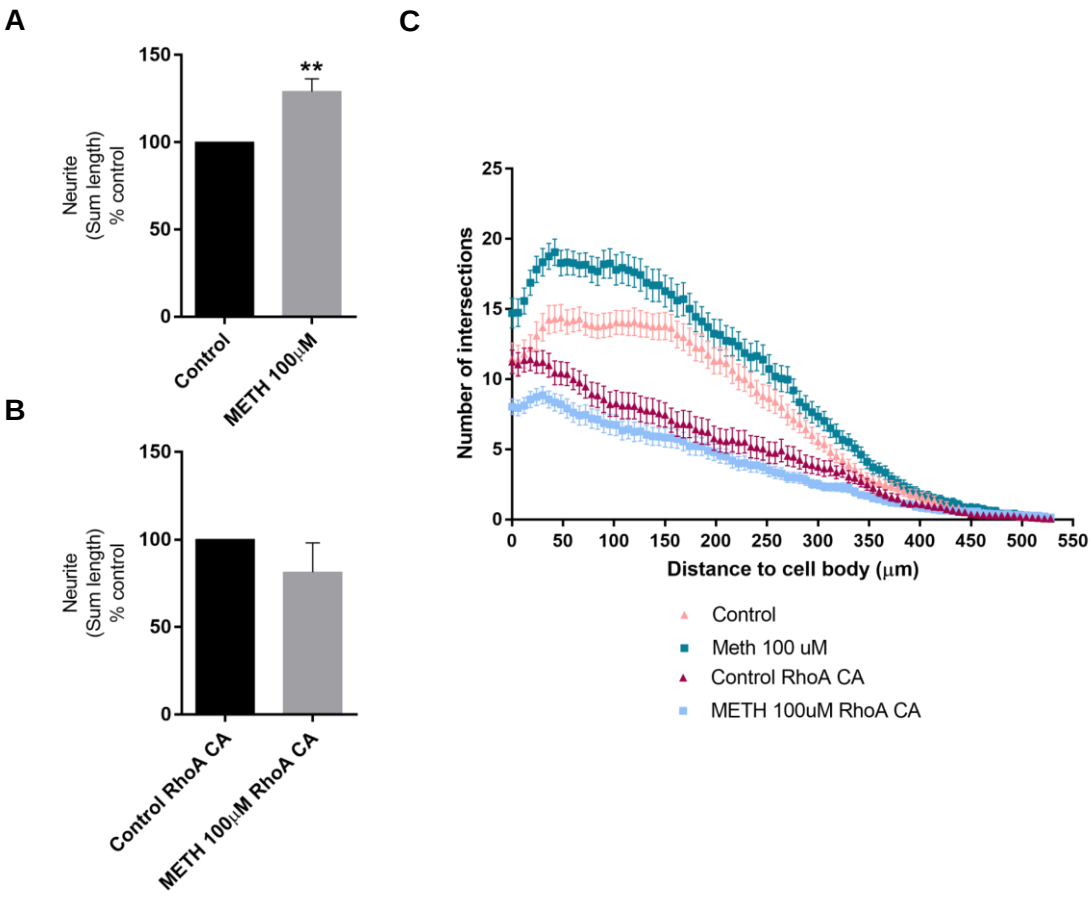


Figure 14. Morphological alterations in response to METH. Primary hippocampal neurons tranfected with GFP or RhoA CA were evaluated for neurite length and branching. Methamphetamine increases neurite length and branching. RhoA CA impairs METH effects on neurite length, which no longer increases. Furthermore, there is a decrease in branching in both control and METH exposed neurons. Statistical analysis was performed using a paired t-test for neuronal length and a two-way ANOVA for neuronal branching.

3.5. Knockdown of Olfm4 protein expression in hippocampal neuronal cultures

To further the possible role of Olfm4 under METH exposure, we silenced this protein in our cultures and evaluated the effect of METH with or without Olfm4 in pathways of interest, which were identified in chapter 3.4.

In a first approach, we infected neurons with lentivirus expressing Olfm4 shRNA or DsRed to assess the protein expression levels of Olfm4. DsRed infected neurons allows us to control for the effects of infection and METH exposure without knocking down Olfm4. Although, we were not yet able to confirm the silencing of Olfm4 protein levels by western blot and therefore to confirm if the shRNA was in fact downregulating Olfm4 protein levels (the antibody initially used is no longer targeting our protein of interest even in positive

controls, another batch will be ordered), we decided to proceed with our study (pending, of course, future confirmation).

Src activation was then reanalysed by western blot to evaluate Src phosphorylation levels in hippocampal neurons infected with lentivirus expressing DsRed or Olfm4 shRNA and exposed or not to METH. Figure 15 represents these preliminary results (n=1), showing a promising decrease in Src phosphorylation levels (as a ratio to the total Src expression) in response to Olfm4 knockdown. Importantly, this may indicate that Olfm4 expression is required for Src activation. Also in this case, at 24h of exposure METH does not seem to affect Src phosphorylation.

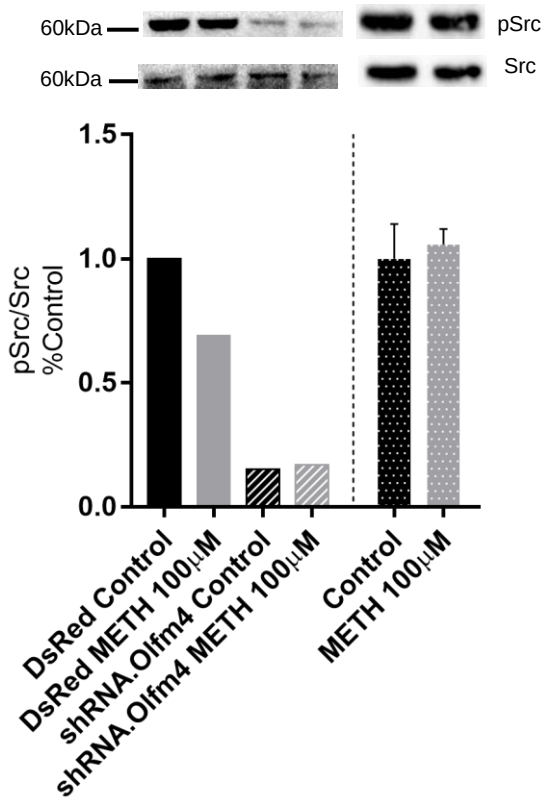


Figure 15. pSrc expression expression in neurons infected with either DsRed or shRNA.Olfm4. METH does not appear to significantly affect the Src phosphorylation in DsRed infected primary hippocampal cultures when compared to control conditions. When knocking down Olfm4, Src phosphorylation decreases in both control and METH exposed neurons. Src acrivaiton in hippocampal neuronal cultures in control vs METH 100μM. Src was used as a loading control. Columns represent one independent experiment.

As mentioned above, Src is involved in modulating MMPs (and in particularly MMP2), which were already reported to be affected by METH (Fernandes et al, 2016; Martins et al, 2011). Therefore, we also became interested in evaluating how silencing Olfm4 could affect METH action over MMP increased release. In order to study the activity of MMPs activity, we collected the cultured condicioned medium of hippocampal neurons infected with lentivirus

expressing Olfm4 shRNA or DsRed, exposed or not to 100 µM METH for 24 hours, and analysed it through a gelatin zymography assay. Surprisingly, knocking down Olfm4 seems to induce an increase in MMP2 activity under METH exposure, although this difference is not statistically significant (figure 16). Although in endothelial cells we have reported a METH induced activation of MMP9 (Fernandes 2016), here, we were not able to detect MMP9 activation signal.

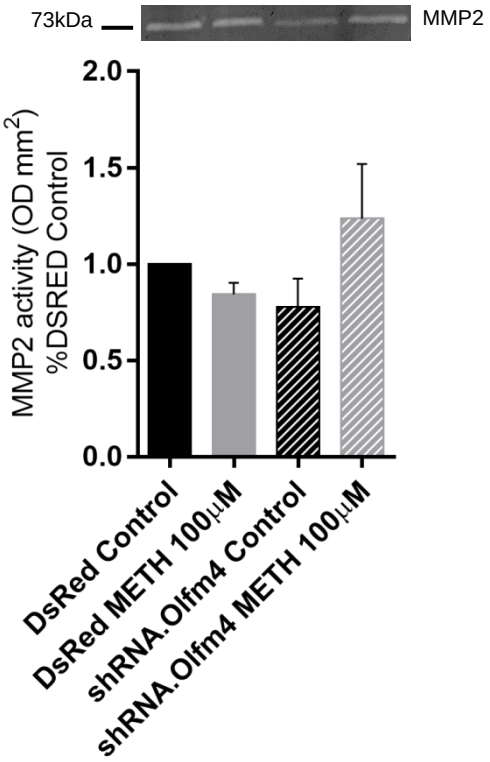


Figure 16. MMP2 activity evaluated in medium collected from neuronal cultures. Conditioned medium of infected 15 DIV hippocampal cultures was collected and processed for a zymography assay. Olfm4 knockdown appears to increase MMP2 activity, when exposed to METH. The results are the average of ±SEM of 4 independent experiments performed in different preparations. Statistical analysis was performed using a one-way ANOVA with Bonferroni post-hoc.

To completely discard a role of Olfm4 in the expression and localization of ZO-1, we further assessed it in neurons infected with lentivirus expressing Olfm4 shRNA or DsRed, exposed or not to METH. As represented in figure 17, it seems that there are no differences between the different conditions. Furthermore, we also analysed ZO-1 expression by immunocytochemistry assays in neurons transfected with Olfm4 shRNA and treated or not with METH. These results are representative since we only analysed 1 independent experiment, and therefore require further experiments.

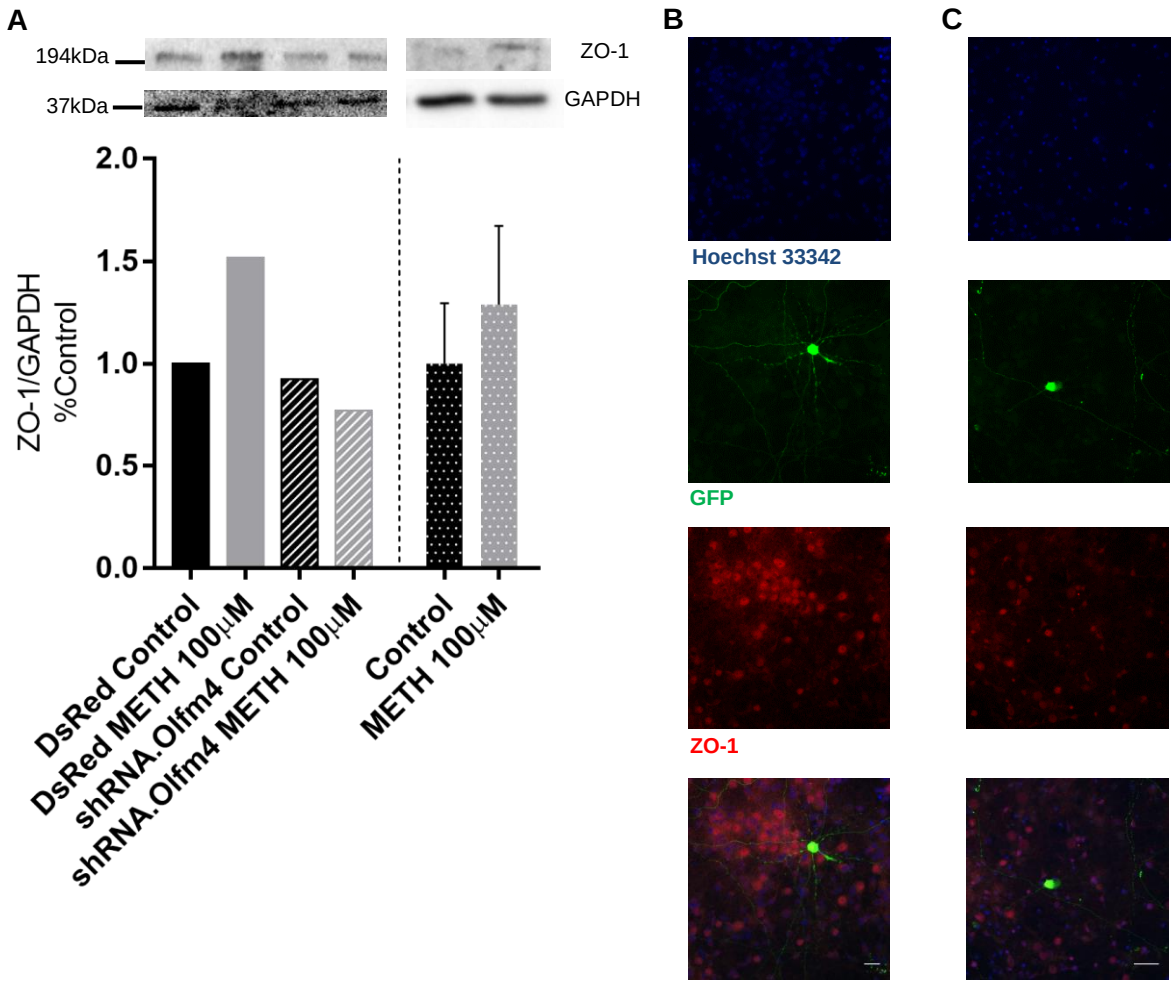


Figure 17. ZO-1 expression in neurons infected with DsRed or shRNA.Olfm4.

(A) METH does not appear to significantly alter ZO-1 expression in hippocampal cultured neurons infected with DsRed. Knocking down Olfm4 does not seem to affect ZO-1 expression in control vs METH exposed neurons. ZO-1 expression in hippocampal neuronal cultures in control vs METH 100µM. GAPDH was used as a loading control. (B) ZO-1 expression in control Venus transfected neurons. (C) ZO-1 expression in METH 100µM Venus transfected neurons.

Chapter 4: Discussion and Conclusion

Neuroinflammation is now seen as a serious consequence of drug abuse. Our aim was to study how methamphetamine might alter the expression of proteins that somehow contribute for neuroinflammation. We showed that Olfm4, a protein that has been linked to inflammatory processes, has its mRNA levels increased in response to METH exposure. Protein levels however, appear to be decreased.

These contradictory results might be explained by cellular regulatory mechanisms, such as miRNA, which function as RNA silencing and in post-transcriptional regulation, potentially leading to mRNA degradation and, therefore, decreasing protein expression. Post-transcriptional mechanisms responsible for mRNA translation into protein are not fully understood and may be accountable for this discrepancy. However, as mentioned earlier, Olfm4 is an extracellular cellular matrix protein which means that it is secreted by cells. One possible explanation for the decreased protein levels might be a METH-induced increase in protein secretion to the extracellular medium, resulting in virtual lower protein levels in the cell extract, and also to increased gene expression as a compensating mechanism. To clarify this issue, we tried to precipitate the protein present in culture medium using the TCA (trichloroacetic acid) method. This protocol uses TCA to precipitate proteins, which after precipitation are resuspended in protein extraction buffer for further analysis by western blot. However, we were not successful in this experiment since we were not able to properly resuspend proteins, and a further approach will include the analysis of the protein secreted using protein lyophilization.

We first evaluated the effect of METH in the mRNA expression of inflammatory factors in neurons (IL-1β, TNF, iNOS, IL-6 and TGF-β), by qPCR. However, we were not able to detect mRNA levels of any of these factors, due to very low expression levels, making it undetectable by qPCR. This may indicate that neurons do not respond directly to METH by producing cytokines. In on-going work at our laboratory we have also seen that the conditioned medium of hippocampal neurons exposed to METH is not able to activate cultured primary microglia cells, indicating that neurons might not produce inflammatory factors in direct response to METH.

Upon this challenge, we decided to shift our focus to extracellular matrix remodeling. Olfm4 has been described as being relevant for tumor growth and matrix shaping (Tomarev and Nakaya, 2009; Zhang et al, 2004) and previous reports from both our and other research groups have shown that METH affects several elements related to these functions (Fernandes et al, 2016; reviewed in Mulholland et al, 2016). As such, we decided to target several of those elements.

Integrins are extensively studied cell adhesion molecules that bind to the extracellular matrix and are involved known to be involved in synaptic plasticity. Previous works have shown that integrins may contribute to psychostimulant-reinstated drug-seeking, by modulating glutamatergic signaling (Wiggins et al, 2011). Laminin was also seen to be decreased after in vivo METH administration and was associated to BBB loss of integrity (Urrutia et al, 2012). Therefore β1 Integrin and Laminin become targets of interest in our study. Unfortunately, the expression of those proteins could not be determined with the antibodies used, and as we were not able to quantify it in control cells; these experiments will need to be repeated to clarify if this is simply due to low expressions levels in our cultures, or if we may need different antibodies. Our results show that methamphetamine does not alter Cldn5 or ZO-1 protein expression in cultured hippocampal neurons. These proteins are classically associated to endothelial cells and BBB function; however they are also expressed in different types of neurons and were reported to be altered in these cells in patients suffering from dementia (Romanitan et al, 2010). Furthermore, our laboratory has recently shown that CLdn5 displays both an altered expression and a significant translocation to cytosol in endothelial cells exposed to METH (Fernandes et al 2016), meaning that although here we do not see an effect at the expression level under METH exposure, we could still find evidence of translocation. For that reason we later tried to evaluate that for ZO-1 through immunocytochemistry, nevertheless we found also no differences in ZO-1 location.

It has been described that MMP2 protein activity increases in response to METH (Fernandes et al 2016; Martins et al, 2011; Mizoguchi et al, 2008). We have assessed the activity of MMPs and it has also been described that MMP9 activity is influenced by Olfm4, since overexpressing this protein results in decreased expression of MMP9. (Park et al, 2012). We have assessed the activity of MMPs in our hippocampal cultures. Interestingly, MMP2 activity in our neuronal cells was not affected by METH, as shown by zymography assay of the culture medium of hippocampal neuronal cultures. However, knocking-down Olfm4 seems to increase MMP2 activity, indicating that Olfm4 expression may have a role on regulating MMP2 activity under METH.

Src family proteins have long been linked to several cellular functions and, among them, neuronal plasticity and extracellular matrix modeling (reviewed in Ohnishi et al, 2011). Of note, our laboratory has recently shown that methylphenidate (MPH), an amphetamine-like stimulant commonly prescribed for attention deficit hyperactivity disorder, induces Rac1/NOX-dependent ROS generation and subsequent c-Src activation (Coelho-Santos et al, 2016). As such, we hypothesized that Src activity could be altered in hippocampal neurons in response to METH exposure. We tested this hypothesis through western blot, quantifying expression of Src and p-Src, which translates into active Src, and through FRET

assay, measuring c-Src activation in response to METH exposure. Our results indicate that methamphetamine increases c-Src activation in a prolonged way. These results might have a broader implication in neuronal response to methamphetamine considering Src pivotal role in modeling the activity of numerous proteins and, therefore, numerous cellular mechanisms.

One specific protein that might be regulated by Src activation is RhoA. This protein activation, as mentioned before, has been linked to a decreased neuronal plasticity. Psychostimulants, like methamphetamine, are known to cause neurite outgrowth and increase the neuronal branching and density of dendritic spines (reviewed in Golden and Russo, 2012). Src activation has been suggested to precede RhoA inactivation, through p190RhoGAP activation (Arthur et al, 2000). In a parallel ongoing work in our laboratory, we have already seen that METH needs to down regulate the activity of RhoA to be able to induce increased morphologic complexity (unpublished data). Although this hypothesis needs to be further explored, we might have unveiled a possible pathway linking Src activation, RhoA diminished activity and higher neurite outgrowth. Schoenwaelder and Burridge (1999) suggested that integrins can transmit signals that lead to rearrangements in the actin cytoskeleton. Arthur et al (2000) suggested that Src activation, and consequent RhoA inactivation, is mediated by extracellular matrix proteins binding to integrins that will outset a signaling cascade that leads to Src activation and RhoA inactivation. Furthermore, our results may also indicate that Olfm4 is involved in Src activation, since reducing Olfm4 expression decreased Src activation, even under METH-exposure. This experiment was performed in only one independent neuronal culture, and therefore requires repetition. Olfm4 has been described to interact with integrins, and is likely that increased Olfm4 secretion could lead to an increased integrin binding, resulting in increased Src activation. Src would then activate p190RhoGAP which, by its turn, would decrease RhoA activity.

To test this theory, our future work will begin by validating Olfm4 knockdown by qPCR, to make sure that we are fact reducing Olfm4 expression in hippocampal neurons. The next step will be to determine if Olfm4 knockdown has an effect in Src and RhoA activation. To do so, we can resort to western blot analyzes or to FRET assays. If our hypothesis is correct, when Olfm4 expression is reduced, we expect to observe a decrease in Src activation under METH exposure, while RhoA activation increases.

Another approach will be to reduce Src expression or inhibit Src activity, and evaluate RhoA activation. This will allow us to stablish a direct link between Src and RhoA activation. To determine if the hypothetical pathway does involve p190RhoGAP activation, we could use the same strategy to assess this protein effect in the signaling pathway.

In summary, we may have uncovered a new pathway that may contribute to the METH-induced neuronal morphological alteration. This pathway involves Src activation, and subsequent RhoA inhibition, which we have previously associated with increased neurite

outgrowth and branching (reviewed in Gonzalez-Billault et al, 2012). This pathway may be linked to our protein of interest Olfm4, since this protein has been shown to regulate integrins, which regulate Src activation, and Olfm4 shRNA decreased Src activation. Our future perspectives are to unravel the role of Olfm4 in this pathway, and dissect the effects in neuronal processes induced by METH, providing a new glimpse into the mechanisms by which METH alters neuronal and brain functioning. Importantly, the effects of Olmf4 in neuronal morphology and regulation of this signaling pathway may be relevant to understand METH addiction.

References

Abbas AK, Lichtman AH, Pillai S (2012) Basic Immunology: Functions and disorders of the immune system. Philadelphia, PA: Elsevier Saunders.

Abbott NJ, Rönnbäck L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 7(1): 41-53.

Albertson TE, Derlet RW, Van Hoozen BE (1999) Methamphetamine and the expanding complications of amphetamines. West J Med. 170(4): 214-219.

Anglin MD, Burke C, Perrochet B, Stamper E, Dawud-Noursi S (2000) History of the Methamphetamine Problem. J Psychoactive Drugs. 32(2): 137-141.

Arias-Carrión O, Stamelou M, Murillo-Rodríguez E, Menéndez-González M, Pöppel E (2010) Dopaminergic reward system: a short integrative review. Int Arch Med. 3:24.

Arthur WT, Petch LA, Burridge K (2000) Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. Curr Biol. 10(12): 719-722.

Asanuma M, Miyazaki I, Higashi Y, Tsuji T, Ogawa N (2004) Specific gene expression and possible involvement of inflammation in methamphetamine-induced neurotoxicity. Ann N Y Acad Sci. 1025:69-75.

Barr AM, Panenka WJ, MacEwan GW, Thornton AE, Lang DJ, Honer WG, Lecomte T (2006) The need for speed: an update on methamphetamine addiction. J Psychiatry Neurosci. 31(5): 301-13.

Basic Principles of RT-qPCR, Thermo Fisher Scientific. https://www.thermofisher.com/pt/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html. Accessed in 05/01/2016.

Belarbi K, Jopson T, Tweedie D, Arellano C, Luo W, Greig NH, Rosi S (2012) TNF-α protein synthesis inhibitor restores neuronal function and reverses cognitive deficits induced by chronic neuroinflammation. J Neuroinflammation. 9:23.

Berridge KC, Kringelbach ML (2008) Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology 199(3): 457-480.

Biber K, Neumann H, Inoue K, Boddeke HWGM (2007) Neuronal ‘On’ and ‘Off’ signals control microglia. Trends Neurosci. 30(11): 596-602.

Bio-Rad Laboratories, Inc (2006). Real-Time PCR Applications Guide.

Bowyer JF, Ali S (2006) High doses of methamphetamine that cause disruption of the blood-brain barrier in limbic regions produce extensive neuronal degeneration in mouse hippocampus. Synapse. 60(7): 521-532.

Bowyer JF, Robinson B, Ali S, Schmued LC (2008) Neurotoxic-related changes in tyrosine hydroxylase, microglia, myelin, and the blood-brain barrier in the caudate-putamen from acute methamphetamine exposure. Synapse 62(3): 193-204.

Brecht ML, O'Brien A, von Mayrhauser C, Anglin MD (2004) Methamphetamine use behaviors and gender differences. Addict Behav. 29(1): 89-106.

Brionne TC, Tesseur I, Masliah E, Wyss-Coray T (2003) Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. Neuron. 40(6): 1133-1145.

Brkic M, Balusu S, Libert C, Vandenbroucke RE (2015) Friends or Foes: Matrix Metalloproteinases and Their Multifaceted Roles in Neurodegenerative Diseases. Mediators Inflamm. 620581.

Buch S, Yao H, Guo M, Mori T, Mathias-Costa B, Singh V, Seth P, Wang J, Su TP (2012) Cocaine and HIV-1 interplay in CNS: Cellular and molecular mechanisms. Curr HIV Res. 10(5): 425-428.

Caldwell J, Dring LG, Williams RT (1972) Metabolism of (14 C) methamphetamine in man, the guinea pig and the rat. Biochem J. 129(1): 11-22.

Chavarria A, Alcocer-Varela J (2004) Is damage in central nervous system due to inflammation? Autoimmun Rev. 3(4): 251-260.

Coelho-Santos V, Socodato R, Portugal C, Leitão RA, Rito M, Barbosa M, Couraud PO, Romero IA, Weksler B, Minshall RD, Fontes-Ribeiro C, Summavielle T, Relvas JB, Silva AP (2016) Methylphenidate-triggered ROS generation promotes caveolae-mediated transcytosis via Rac1 signaling and c-Src-dependent caveolin-1 phosphorylation in human brain endothelial cells. Cell Mol Life Sci.

Cruickshank CC, Dyer KR (2009) A review of the clinical pharmacology of methamphetamine. Addiction 104(7): 1085-1099.

Cunha-Oliveira T, Rego AC, Oliveira CR (2008) Cellular and molecular mechanisms involved in the neurotoxicity of opioid and psychostimulant drugs. Brain Res Rev. 58(1): 192-208.

Darke S, Kaye S, McKetin R, Duflou J (2008) Major physical and psychological harms of methamphetamine use. Drug Alcohol Rev. 27(3):253-262.

Davachi L (2006) Item, context and relational episodic encoding in humans. Curr Opin Neurobiol. 16(6): 693-700.

Davidow JY, Foerde K, Galván A, Shohamy D (2016) An Upside to Reward Sensitivity: The Hippocampus Supports Enhanced Reinforcement Learning in Adolescence. Neuron. 92(1): 93-99.

Durieux PF, Bearzatto B, Guiducci S, Buch T, Waisman A, Zoli M, Schiffmann SN, d'Exaerde AK (2009) D2R striatopallidal neurons inhibit both locomotor and drug reward processes. Nat Neurosci. 12(4): 393-395.

EBF3 Gene, GeneCards. http://www.genecards.org/cgi-bin/carddisp.pl?gene=EBF3. Acessed in 17/03/2016.

European Monitoring Centre for Drugs and Drug Addiction (2010), Problem amphetamine and methamphetamine use in Europe, EMCDDA Papers, Publications Office of the European Union, Luxembourg.

European Monitoring Centre for Drugs and Drug Addiction (2014), European Drug Report 2014: Trends and developments, EMCDDA Papers, Publications Office of the European Union, Luxembourg.

Fernandes S, Salta S, Bravo J, Silva AP, Summavielle T (2014) Acetyl-L-Carnitine Prevents Methamphetamine-Induced Structural Damage on Endothelial Cells via ILK-Related MMP-9 Activity. Mol Neurobiol. 53(1): 408-422.

Fernandes S, Salta S, Bravo J, Silva AP, Summavielle T (2016) Acetyl-L-Carnitine Prevents Methamphetamine-Induced Structural Damage on Endothelial Cells via ILK-Related MMP-9 Activity. Mol Neurobiol. 53(1):408-422.

Final act of the United Nations conference for the adoption of a protocol on psychotropic substances, 1971.

Fleckenstein AE, Hanson GR (2003) Impact of psychostimulants on vesicular monoamine transporter function. Eur J Pharmacol. 479(1-3): 283-289.

Fornai F, Lenzi P, Gesi M, Soldani P, Ferrucci M, Lazzeri G, Capobianco L, Battaglia G, De Blasi A, Nicoletti F, Paparelli A (2004) Methamphetamine produces neuronal inclusions in the nigrostriatal system and in PC12 cells. J Neurochem. 88(1): 114-123.

Fornai F, Lenzi P, Lazzeri G, Ferrucci M, Fulceri F, Giorgi FS, Falleni A, Ruggieri S, Paparelli A (2007) Fine ultrastructure and biochemistry of PC12 cells: a comparative approach to understand neurotoxicity. Brain Res. 1129(1): 174-190.

Gold MS, Kobeissy FH, Wang KK, Merlo LJ, Bruijnzeel AW, Krasnova IN, Cadet JL (2009) Methamphetamine- and trauma-induced brain injuries: comparative cellular and molecular neurobiological substrates. Biol Psychiatry. 66(2): 118-127.

Golden SA, Russo SJ (2012) Mechanisms of psychostimulant-induced structural plasticity. Cold Spring Harb Perspect Med. 2(10). pii: a011957.

Gonçalves J, Baptista S, Martins T, Milhazes N, Borges F, Ribeiro CF, Malva JO, Silva AP (2009) Methamphetamine-induced neuroinflammation and neuronal dysfunction in the mice hippocampus: preventive effect of indomethacin. Eur J Neurosci. 31(2): 315-326.

Gonzalez-Billault C, Muñoz-Llancao P, Henriquez DR, Wojnacki J, Conde C, Caceres A (2012) The role of small GTPases in neuronal morphogenesis and polarity. Cytoskeleton. 69(7): 464-485.

Govek EE, Newey SE, Van Aelst L (2005) The role of the Rho GTPases in neuronal development. Genes Dev. 19(1): 1-49.

Hartzell C, Ksionda O, Lemmens E, Coakley K, Yang M, Dail M, Harvey RC, Govern C, Bakker J, Lenstra TL, Ammon K, Boeter A, Winter SS, Loh M, Shannon K, Chakraborty AK, Wabl M, Roose JP (2013) Dysregulated RasGRP1 responds to cytokine receptor input in T cell leukemogenesis. Sci Signal. 6(268): ra21.

Heneka MT, Feinstein DL (2001) Expression and function of inducible nitric oxide synthase in neurons. J Neuroimmunol. 114(1-2): 8-18.

Homer BD, Solomon TM, Moeller RW, Mascia A, DeRaleau L, Halkitis PN (2008) Methamphetamine abuse and impairment of social functioning: a review of the underlying neurophysiological causes and behavioral implications. Psychol Bull. 134(2): 301-310.

Hyman S, Malenka R, Nestler E (2006) Neural Mechanisms of Addiction: The Role of Reward-Related Learning and Memory. Annu Rev Neurosci. 29: 565-598.

Jaffe AB, Hall A (2005) Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 21: 247-69.

Jedynak JP, Uslaner JM, Esteban JA, Robinson TE (2007) Methamphetamine-induced structural plasticity in the dorsal striatum. Eur J Neurosci. 25(3): 847-853.

Jurgens HA, Johnson RW (2012) Dysregulated neuronal–microglial cross-talk during aging, stress and inflammation. Exp Neurol. 233(1): 40-48.

Kalda A, Heidmets LT, Shen HY, Zharkovsky A, Chen JF (2007) Histone deacetylase inhibitors modulates the induction and expression of amphetamine-induced behavioral sensitization partially through an associated learning of the environment in mice. Behav Brain Res. 181(1): 76-84.

Kalivas PW, Volkow ND (2005) The neural basis of addiction: a pathology of motivation and choice. Am J Psychiatry. 162(8): 1403-1413.

Kenny PJ (2007) Brain reward systems and compulsive drug use. Trends Pharmacol Sci. 28(3): 135-141.

Kish SJ (2008) Pharmacologic mechanisms of crystal meth. CMAJ. 178(13): 1679–1682.

Kishimoto T (2010) IL-6: from its discovery to clinical applications. Int Immunol. 22(5): 347-352.

Kiyatkin EA, Sharma HS (2009) Acute methamphetamine intoxication: brain hyperthermia, blood-brain barrier, brain edema, and morphological cell abnormalities. Int Rev Neurobiol. 88: 65-100.

Koob GF (2006) The neurobiology of addiction: a neuroadaptational view relevant for diagnosis. Addiction. 101(1): 23-30.

Koob GF, Le Moal M (2001) Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology. 24(2): 97-129.

Koob GF, Le Moal M (2005) Plasticity of reward neurocircuitry and the “dark side” of drug addiction. Nat Neurosci. 8(11): 1442-1444.

Koob GF, Volkow ND (2010) Neurocircuitry of Addiction. Neuropsychopharmacology. 35(1): 217-238.

Kozera, Rapacz (2013) Reference genes in real-time PCR. J Appl Genet. 54(4): 391–406.

Krasnova IN, Cadet JL (2009) Methamphetamine toxicity and messengers of death. Brain Res Rev. 60(2): 379-407.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 19(8): 312-318.

Kuo L, Chang HC, Leu TH, Maa MC, Hung WC (2006) Src oncogene activates MMP-2 expression via the ERK/Sp1 pathway. J Cell Physiol. 207(3): 729-734.

Langhammer CG, Previtera ML, Sweet ES, Sran SS, Chen M, Firestein BL (2010) Automated Sholl analysis of digitized neuronal morphology at multiple scales: Whole cell Sholl analysis versus Sholl analysis of arbor subregions. Cytometry A. 77(12): 1160-1168.

Lee DC, Ruiz CR, Lebson L, Selenica ML, Rizer J, Hunt JB Jr, Rojiani R, Reid P, Kammath S, Nash K, Dickey CA, Gordon M, Morgan D (2013) Aging enhances classical activation but mitigates alternative activation in the central nervous system. Neurobiol Aging. 34(6): 1610-1620.

Lee YW, Hennig B, Yao J, Toborek M (2001) Methamphetamine induces AP-1 and NF-kappaB binding and transactivation in human brain endothelial cells. J Neurosci Res. 66(4): 583-591.

Levite M (2008) Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. Curr. Opin. Pharmacol. 8(4): 460-471.

Lindquist R, Niesner R (2015) Intravital FRET: comprehending life at single-molecule level. Focus on “A practical method for monitoring FRET-based biosensors in living animals using two-photon microscopy”. Am J Physiol Cell Physiol. 309(11): C722-723.

Liu W, Lee HW, Liu Y, Wang R, Rodgers GP (2010) Olfactomedin 4 is a novel target gene of retinoic acids and 5-aza-2'-deoxycytidine involved in human myeloid leukemia cell growth, differentiation, and apoptosis. Blood. 116(23): 4938-4947.

Liu W, Yan M, Sugui JA, Li H, Xu C, Joo J, Kwon-Chung KJ, Coleman WG, Rodgers GP (2013) Olfm4 deletion enhances defense against Staphylococcus aureus in chronic granulomatous disease. J Clin Invest. 123(9): 3751-3755.

Loftis JM, Janowsky A (2014) Neuroimmune Basis of Methamphetamine Toxicity. Int Rev Neurobiol. 118: 165-197.

Lyman M, Lloyd DG, Ji X, Vizcaychipi MP, Ma D (2014) Neuroinflammation: the role and consequences. Neurosci Res. 79: 1-12.

Lynch J, House MA (1992) Cardiovascular effects of methamphetamine. J Cardiovasc Nurs. 6(2): 12-18.

Marienfeld R, May MJ, Berberich I, Serfling E, Ghosh S, Neumann M (2003) RelB forms transcriptionally inactive complexes with RelA/p65. J Biol Chem. 278(22):19852-19860.

Marshall J, Belcher AM, Feinstein EM, O'Dell S (2007) Methamphetamine-induced neural and cognitive changes in rodents. Addiction. 102 Suppl 1:61-69.

Marshall J, O'Dell S (2012) Methamphetamine influences on brain and behavior: unsafe at any speed? Trends Neurosci. 35(9): 536-545.

Martins T, Baptista S, Gonçalves J, Leal E, Milhazes N, Borges F, Ribeiro CF, Quintela O, Lendoiro E, López-Rivadulla M, Ambrósio AF, Silva AP (2011) Methamphetamine transiently increases the blood–brain barrier permeability in the hippocampus: Role of tight junction proteins and matrix metalloproteinase-9. Brain Res. 1411: 28-40.

Masson J, Sagné C, Hamon M, El Mestikawy S (1999) Neurotransmitter transporters in the central nervous system. Pharmacol Rev. 51(3): 439-464.

MATLAB 6.1, The MathWorks Inc., Natick, MA, 2000

McCann UD, Kuwabara H, Kumar A, Palermo M, Abbey R, Brasic J, Ye W, Alexander M, Dannals RF, Wong DF, Ricaurte GA (2008) Persistent cognitive and dopamine transporter deficits in abstinent methamphetamine users. Synapse. 62(2): 91-100.

McCann UD, Wong DF, Yokoi F, Villemagne V, Dannals RF, Ricaurte GA (1998) Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinone users: evidence from positron emission tomography studies with [11C]WIN-35,428. J Neurosci. 18(20): 8417-8422.

Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, Unser M (2004) Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A. 58(2): 167-176.

Meredith CW, Jaffe C, Ang-Lee K, Saxon AJ (2005) Implications of chronic methamphetamine use: a literature review. Harv Rev Psychiatry. 13(3): 141-154.

Methamphetamine, PubChem. http://pubchem.ncbi.nlm.nih.gov/compound/methamphetamine#section=Top. Acessed in 08/01/2016.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev. 78(1):189-225.

Mizoguchi H, Yamada K, Nabeshima T (2008) Neuropsychotoxicity of abused drugs: involvement of matrix metalloproteinase-2 and -9 and tissue inhibitor of matrix metalloproteinase-2 in methamphetamine-induced behavioral sensitization and reward in rodents. J Pharmacol Sci. 106(1): 9-14.

Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, ZhaoZ, Toga AW, Jacobs RE, Liu CY, Amezcua L, Harrington MG, Chui HC, Law M, Zlokovic BV (2015) Blood-Brain Barrier Breakdown in the Aging Human Hippocampus. Neuron. 85(2): 296-302.

Mott RT, Ait-Ghezala G, Town T, Mori T, Vendrame M, Zeng J, Ehrhart J, Mullan M, Tan J (2004) Neuronal expression of CD22: novel mechanism for inhibiting microglial proinflammatory cytokine production. Glia. 46(4): 369-379.

Mulholland PJ, Chandler LJ, Kalivas PW (2016) Signals from the Fourth Dimension Regulate Drug Relapse. Trends Neurosci. 39(7):472-485.

Murphy P, Grondin J, Altares M (1995) Induction of interleukin-6 in axotomized sensory neurons. J. Neurosci. 15(7Pt 2): 5130-5138.

Murray JB (1998) Psychophysiological Aspects of Amphetamine-Methamphetamine Abuse. J Psychol 132(2): 227-237.

Nakamura M, Murray ME, Lin WL, Kusaka H, Dickson DW (2014) Optineurin immunoreactivity in neuronal and glial intranuclear inclusions in adult-onset neuronal intranuclear inclusion disease. Am J Neurodegener Dis. 3(2): 93-102.

Narita M, Miyatake M, Narita M, Shibasaki M, Shindo K, Nakamura A, Kuzumaki N, Nagumo Y, Suzuki T (2006) Direct evidence of astrocytic modulation in the development of rewarding effects induced by drugs of abuse. Neuropsychopharmacology. 31(11): 2476-2488.

Nesse RM, Berridge KC (1997) Psychoactive drug use in evolutionary perspective. Science. 278(5335): 63-66.

Nikaido T, Akiyama M, Moriya T, Shibata S (2001) Sensitized increase of period gene expression in the mouse caudate/putamen caused by repeated injection of methamphetamine. Mol Pharmacol. 59(4): 894-900.

Nobes CD, Hall A (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 81(1): 53-62.

Nordahl TE, Salo R, Leamon M (2003) Neuropsychological Effects of Chronic Methamphetamine Use on Neurotransmitters and Cognition: A Review. J Neuropsychiatry Clin Neurosci. 15(3): 317-325.

Numachi Y, Yoshida S, Yamashita M, Fujiyama K, Naka M, Matsuoka H, Sato M, Sora I (2004) Psychostimulant alters expression of DNA methyltransferase mRNA in the rat brain. Ann N Y Acad Sci. 1025: 102-109.

O'Callaghan JP, Sriram K, Miller DB (2008) Defining "neuroinflammation". Ann N Y Acad Sci. 1139:318-330.

Ohnishi H, Murata Y, Okazawa H, Matozaki T (2011) Src family kinases: modulators of neurotransmitter receptor function and behavior. Trends Neurosci. 34(12): 629-637.

Olivenza R, Moro MA, Lizasoain I, Lorenzo P, Fernández AP, Rodrigo J, Boscá L, Leza JC (2000) Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. J Neurochem. 74(2): 785-791.

OPTN Gene, Genecards. http://www.genecards.org/cgi-bin/carddisp.pl?gene=OPTN. Acessed in 10/03/2016.

Ouyang M, Sun J, Chien S, Wang Y (2008) Determination of hierarchical relationship of Src and Rac at subcellular locations with FRET biosensors. Proc Natl Acad Sci U S A. 105(38): 14353-14358.

Pacholczyk T, Blakely RD, Amara SG (1991) Expression cloning of an Antidepressant antidepressant-sensitive noradrenaline transporter. Nature. 350(6316): 350-354.

Palmer AE, Jin C, Reed JC, Tsien RY (2004) Bcl-2-mediated alterations in endoplasmic reticulum Ca2+ analysed with an improved genetically encoded fluorescent sensor. Proc Natl Acad Sci U S A. 101(50): 17404-17409.

Panenka WJ, Procyshyn RM, Lecomte T, MacEwan GW, Flynn SW, Honer WG, Barr AM (2013) Methamphetamine use: A comprehensive review of molecular, preclinical and clinical findings. Drug Alcohol Depend. 129(3): 167-179.

Park KS, Kim KK, Piao ZH, Kim MK, Lee HJ, Kim YC, Lee KS, Lee JH, Kim KE (2012) Olfactomedin 4 suppresses tumor growth and metastasis of mouse melanoma cells through downregulation of integrin and MMP genes. Mol Cells. 34(6): 555-561.

Perez Jr. JA, Arsura EL, Strategos S (1999) Methamphetamine-related stroke: four cases. J Emerg Med. 17(3): 469-471.

Persidsky Y, Ramirez SH, Haorah J, Kanmogne GD (2006) Blood-brain barrier: Structural components and function under physiologic and pathologic conditions. J Neuroimmune Pharmacol. 1(3): 223-236.

Pertz OC, Wang Y, Yang F, Wang W, Gay LJ, Gristenko MA, Clauss TR, Anderson DJ, Liu T, Auberry KJ, Camp DG 2nd, Smith RD, Klemke RL (2008) Spatial mapping of the neurite and soma proteomes reveals a functional Cdc42/Rac regulatory network. Proc Natl Acad Sci U S A. 105(6): 1931-1936.

Petty MA, Lo EH (2002) Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation. Prog Neurobiol. 68(5): 311-323.

Phillips AG, Vacca G, Ahn S (2008) A top-down perspective on dopamine, motivation and memory. Pharmacol Biochem Behav. 90(2): 236-249.

Pocock JM, Kettenmann H (2007) Neurotransmitter receptors on microglia. Trends Neurosci. 30(10): 527-535.

Ramesh G, MacLean AG, Philipp MT (2013) Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. Mediators Inflamm. 2013:480739.

Ramirez SH, Potula R, Fan S, Eidem T, Papugani A, Reichenbach N, Dykstra H, Weksler BB, Romero IA, Couraud PO, Persidsky Y (2009) Methamphetamine disrupts blood-brain barrier function by induction of oxidative stress in brain endothelial cells. J Cereb Blood Flow Metab. 29(12): 1933-1945.

RASGRP1 Gene, GeneCards. http://www.genecards.org/cgi-bin/carddisp.pl?gene=RASGRP1&keywords=rasgrp1. Acessed in 16/03/2016.

Renthal W, Carle TL, Maze I, Covington HE 3rd, Truong HT, Alibhai I, Kumar A, Montgomery RL, Olson EN, Nestler EJ (2008) Delta FosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. J Neurosci. 28(29): 7344-7349.

Riddle EL, Fleckenstein AE, Hanson GR (2006) Mechanisms of methamphetamine-induced dopaminergic neurotoxicity. AAPS J. 8(2): E413-E418.

Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell. 70(3): 389-399.

Robinson TE, Berridge KC (2008) The incentive sensitization theory of addiction: some current issues. Philos Trans R Soc Lond B Biol Sci. 363(1507): 3137-3146.

Rusanescu G, Qi H, Thomas SM, Brugge JS, Halegoua S (1995) Calcium influx induces neurite growth through a Src-Ras signaling cassette. Neuron. 15(6): 1415-1425.

Russo SJ, Nestler EJ (2013) The brain reward circuitry in mood disorders. Nat Rev Neurosci. 14(9): 609-625.

Salter MW, Beggs S (2014) Sublime microglia: expanding roles for the guardians of the CNS. Cell. 158(1): 15-24.

Schepers RJ, Oyler JM, Joseph Jr, RE, Cone EJ, Moolchan ET, Huestis MA (2003) Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. Clin Chem. 49(1): 121-132.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods. 9(7): 676-682.

Schoenwaelder SM, Burridge K (1999) Bidirectional signaling between the cytoskeleton and integrins. Curr Opin Cell Biol. 11(2): 274-286.

Schultz W (2007) Behavioral dopamine signals. Trends Neurosci. 30(5): 203-210.

Scott JC, Woods SP, Matt GE, Meyer RA, Heaton RK, Atkinson JH, Grant I (2007) Neurocognitive effects of methamphetamine: a critical review and meta-analysis. Neuropsychol Rev. 17(3): 275-297.

Sekine Y, Minabe Y, Ouchi Y, Takei N, Iyo M, Nakamura K, Suzuki K, Tsukada H, Okada H, Yoshikawa E, Futatsubashi M, Mori N (2003) Association of dopamine transporter loss in the orbitofrontal and dorsolateral prefrontal cortices with methamphetamine-related psychiatric symptoms. Am J Psychiatry. 160(9): 1699-1701.

Sekine Y, Ouchi Y, Sugihara G, Takei N, Yoshikawa E, Nakamura K, Iwata Y, Tsuchiya KJ, Suda S, Suzuki K, Kawai M, Takebayashi K, Yamamoto S, Matsuzaki H, Ueki T, Mori N, Gold MS, Cadet JL (2008) Methamphetamine causes microglial activation in the brains of human abusers. J Neurosci. 28(22): 5756-5761.

Sekine Y, Ouchi Y, Takei N, Yoshikawa E, Nakamura K, Futatsubashi M, Okada H, Minabe Y, Suzuki K, Iwata Y, Tsuchiya KJ, Tsukada H, Iyo M, Mori N (2006) Brain serotonin transporter density and aggression in abstinent methamphetamine abusers. Arch Gen Psychiatry. 63(1): 90-100.

Shabab T, Khanabdali R, Moghadamtousi SZ, Kadir HA, Mohan G (2016) Neuroinflammation pathways: a general review. Int J Neurosci. 9: 1-10.

Shaftel SS, Kyrkanides S, Olschowka JA, Miller JN, Johnson RE, O'Banion MK (2007) Sustained hippocampal IL-1 beta overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. J Clin Invest. 117(6): 1595-1604.

Sharma HS, Kiyatkin EA (2009) Rapid morphological brain abnormalities during acute methamphetamine intoxication in the rat: an experimental study using light and electron microscopy. J Chem Neuroanat. 37(1): 18-32.

Shih VF, Davis-Turak J, Macal M, Huang JQ, Ponomarenko J, Kearns JD, Yu T, Fagerlund R, Asagiri M, Zuniga EI, Hoffmann A (2012) Control of RelB during dendritic cell activation integrates canonical and noncanonical NF-κB pathways. Nat Immunol. 13(12): 1162-1170.

Socodato R, Portugal CC, Canedo T, Domith I, Oliveira NA, Paes-de-Carvalho R, Relvas JB, Cossenza M (2014) c-Src deactivation by the polyphenol 3-O-caffeoylquinic acid abrogates reactive oxygen species-mediated glutamate release from microglia and neuronal excitotoxicity. Free Radic Biol Med. 79: 45-55.

Squire LR, Stark CE, Clark RE (2004) The medial temporal lobe. Annu Rev Neurosci. 27: 279-306.

Streit WJ (2002) Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40(2): 133-139.

Streit WJ, Kincaid-Colton CA (1995) The brain's immune system. Sci Am. 273(5): 54-61.

Sudhakar C, Nagabhushana A, Jain N, Swarup G (2009) NF-kappaB mediates tumor necrosis factor alpha-induced expression of optineurin, a negative regulator of NF-κB. PLoS One 4(4): e5114.

Sulzer D, Sondersb MS, Poulsen NW, Galli A (2005) Mechanisms of neurotransmitter release by amphetamines: A review. Prog Neurobiol. 75(6): 406-433.

Terrando N, Yang T, Ryu JK, Newton PT, Monaco C, Feldmann M, Ma D, Akassoglou K, Maze M (2014) Stimulation of the α7 Nicotinic Acetylcholine Receptor Protects against neuroinflammation after Tibia Fracture and Endotoxemia in Mice. Mol Med. 20: 667-675.

TGFB1 Gene, GeneCards. http://www.genecards.org/cgi-bin/carddisp.pl?gene=TGFB1. Acessed in 17/03/2016.

Thomas DM, Walker PD, Benjamins JA, Geddes TJ, Kuhn DM (2004) Methamphetamine neurotoxicity in dopamine nerve endings of the striatum is associated with microglial activation. J Pharmacol Exp Ther. 311(1): 1-7.

Thompson PM, Hayashi KM, Simon SL, Geaga JA, Hong MS, Sui Y, Lee JY, Toga AW, Ling W, London ED (2004) Structural abnormalities in the brains of human subjects who use methamphetamine. J Neurosci. 24(26): 6028-6036.

Tomarev SI, Nakaya N (2009) Olfactomedin domain-containing proteins: possible mechanisms of action and functions in normal development and pathology. Mol Neurobiol. 40(2):122-138.

Turowski P, Kenny BA (2015) The blood-brain barrier and methamphetamine: open sesame? Front Neurosci. 9:156.

UniProtKB - Q96CV9 (OPTN_HUMAN), Uniprot. http://www.uniprot.org/uniprot/Q96CV9#section_comments. Acessed in 10/03/2016.

United Nations Office on Drugs and Crime, World Drug Report 2014 (United Nations publication, Sales No. E.14.XI.7).

Urrutia A, Rubio-Araiz A, Gutierrez-Lopez MD, ElAli A, Hermann DM, O'Shea E, Colado MI (2012) A study on the effect of JNK inhibitor, SP600125, on the disruption of blood-brain barrier induced by methamphetamine. Neurobiol Dis. 50:49-58.

Vallone D, Picetti R, Borrelli E (2000) Structure and function of dopamine receptors. Neurosci Biobehav Rev. 24(1): 125-132.

Volkow N, Li TK (2005) The neuroscience of addiction. Nat Neurosci. 8(11): 1429-1430.

Volkow ND, Chang L, Wang GJ, Fowler JS, Franceschi D, Sedler M, Gatley SJ, Miller E, Hitzemann R, Ding YS, Logan J (2001a). Loss of dopamine transporters in methamphetamine abusers recovers with protracted abstinence. J Neurosci. 21(23): 9414-9418.

Volkow ND, Chang L, Wang GJ, Fowler JS, Franceschi D, Sedler MJ, Gatley SJ, Hitzemann R, Ding YS, Wong C, Logan J (2001b). Higher cortical and lower subcortical metabolism in detoxified methamphetamine abusers. Am J Psychiatry. 158(3): 383-389.

Volkow ND, Chang L, Wang GJ, Fowler JS, Leonido-Yee M, Franceschi D, Sedler MJ, Gatley SJ, Hitzemann R, Ding YS, Logan J, Wong C, Miller EN (2001c). Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. Am J Psychiatry. 158(3): 377-382.

Volkow ND, Li TK (2004) Drug addiction: the neurobiology of behavior gone awry. Nat Rev Neurosci. 5(12): 963-970.

Volkow ND, Wang GJ, Fowler JS, Tomasi D (2012) Addiction Circuitry in the Human Brain. Annu Rev Pharmacol Toxicol. 52: 321-336.

Wang GJ, Volkow ND, Chang L, Miller E, Sedler M, Hitzemann R, Zhu W, Logan J, Ma Y, Fowler JS (2004) Partial recovery of brain metabolism in methamphetamine abusers after protracted abstinence. Am J Psychiatry. 161(2): 242-248.

Wang Z, Gerstein M and Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 10(1): 57-63.

Weisheit R, White W (2009) Methamphetamine: Its History, Pharmacology and Treatment. USA: Hazelden Publishing.

Wearne SL, Rodriguez A, Ehlenberger DB, Rocher AB, Henderson SC, Hof PR (2005) New techniques for imaging, digitization and analysis of three-dimensional neural morphology on multiple scales. Neuroscience. 136(3): 661-680.

Wiggins A, Smith RJ, Shen H, Kalivas PW (2011) Integrins Modulate Relapse to Cocaine-Seeking. J Neurosci. 31(45): 16177-16184.

Wise RA (2009) Roles for nigrostriatal - not just mesocorticolimbic - dopamine in reward and addiction. Trends Neurosci. 32(10): 517-524.

Yamamoto BK, Moszczynska A, Gudelsky GA (2010) Amphetamine toxicities: classical and emerging mechanisms. Ann N Y Acad Sci. 1187: 101-121.

Yoshizaki H, Ohba Y, Kurokawa K, Itoh RE, Nakamura T, Mochizuki N, Nagashima K, Matsuda M (2003) Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. J Cell Biol. 162(2): 223-232.

YWHAZ Gene, GeneCards. http://www.genecards.org/cgi-bin/carddisp.pl?gene=YWHAZ. Acessed in 16/03/2016

Zhang J, Liu WL, Tang DC, Chen L, Wang M, Pack SD, Zhuang Z, Rodgers GP (2002) Identification and characterization of a novel member of olfactomedin-related protein family, hGC-1, expressed during myeloid lineage development. Gene 283(1-2): 83-93.

Zhang X, Huang Q, Yang Z, Li Y, Li CY (2004) GW112, a novel antiapoptotic protein that promotes tumor growth. Cancer Res. 64(7): 2474-2481.

Zhao LY, Niu Y, Santiago A, Liu J, Albert SH, Robertson KD, Liao D (2006) An EBF3-mediated transcriptional program that induces cell cycle arrest and apoptosis. Cancer Res. 66(19): 9445-9452.

Zweben JE, Cohen JB, Christian D, Galloway GP, Salinardi M, Parent D, Iguchi M (2004) Psychiatric symptoms in methamphetamine users. Am J Addict. 13(2): 181-90.