Ana Rita das Neves Lagarto Bento

O efeito da metanfetamina na neurogénese da zona subventricular: morte celular, proliferação e diferenciação.

Universidade de Coimbra
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Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Biomédica, com especialização em Neurociências.

Este trabalho foi realizado no Centro de Neurociências e Biologia Celular de Coimbra e na Faculdade de Medicina da Universidade de Coimbra, sob a orientação da Doutora Fabienne Agasse e co-orientação da Doutora Ana Paula da Silva Martins.

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Agradecimentos

À Doutora Fabienne Agasse e à Doutora Ana Paula Silva por me terem orientado e supervisionado o trabalho que realizei durante a cadeira de Projecto, por me terem ensinado e ajudado a construir o trabalho, pela amizade, simpatia e paciência apoiando sempre nos bons e maus momentos.

Ao Professor Doutor João Malva pelo apoio e críticas que ajudaram a desenvolver o trabalho.

Ao Professor Doutor Miguel Morgado, coordenador da cadeira de Projecto em que se insere a dissertação da Tese de Mestrado, que promoveu a possibilidade de realizar o trabalho e pelo apoio, simpatia e disponibilidade.

À minha família que constituiu o meu maior apoio neste período, em especial ao meu pai, à minha irmã e à minha mãe que é o meu exemplo de luta, esforço e dedicação.

Aos amigos que me apoiaram nesta fase e que me acompanham até hoje, obrigada pelos conselhos, pelos sorrisos, por tudo!

Aos colegas de trabalho por toda a ajuda, pela simpatia, apoio e críticas, em especial à Joana Gonçalves, Sofia Baptista, Sofia Grade e Alexandra.

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Abbreviations

BrdU	5-bromo-2'-deoxyuridine
DCX	doublecortin
DNA	deoxyribonucleic acid
DG	dentate gyrus
EGF	epidermal growth factor
FGF-2	fibroblast growth factor-2
ICC	immunocytochemistry
JNK	c-Jun-NH_2-terminal kinase
NeuN	neuronal nuclear protein
METH	methamphetamine
P-SAPK/JNK	phosphorylated form of stress activated protein kinase/c-Jun-NH_2-terminal kinase
PFA	paraformaldehyde
PI	propidium idodide
SFM	serum-free culture medium
SGZ	subgranular zone
SOX-2	sex-determining region Y related high-mobility box gene 2
SVZ	subventricular zone
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labelling
ZVAD	z-Val-Ala-DL-Asp (OMe)-fluoromethylketone

Abstract

Methamphetamine (METH) is a highly toxic and addictive psychostimulant widely consumed over the world which constitutes the second drug most abused after cannabis. METH abusers show brain abnormalities in function and in structure. Moreover, impairment in memory often observed in METH abusers suggests that, besides the toxic effect, METH may also alter neurogenesis. The born of new neurons takes place in the adult mammalian brain, including in humans. Indeed, hippocampal neurogenesis is associated with improved capacity to memorize. Accordingly, in animals injected with METH, the proliferation and genesis of granule cells are decreased in the dentate gyrus of the hippocampus and might account for the decrease in memory function observed in METH users.

The subventricular zone (SVZ) is the major neurogenic site of the mammalian brain and contains stem cells. New neurons produced in the SVZ migrate through the rostral migratory stream and add to the olfactory bulb. After differentiation into interneurons, the new added cells improve olfactory function. Interestingly, following several brain damages, such as ischemia, epilepsy, head trauma, neuronal and glial cells degeneration, proliferation increases in the SVZ. Newborn cells are able to migrate out of the SVZ towards injured site. The SVZ represents a major pool of repairing cells in the adult brain. However, little is known about the effect of METH on SVZ stem cells dynamic and neurogenesis.

We undertook this work in order to disclose the effects of METH on neurogenesis in SVZ cultures. SVZ neurospheres were cultured from early postnatal mice and subjected to growing doses of METH while in free floating or 48 hours following plating. We observed that METH at concentrations of and above 100 µM increases cell death by both necrosis and apoptosis in 24 hours treated cultures. Moreover, METH does not preferentially trigger the death of doublecortin neuroblasts. However, stem/progenitor cells expressing SOX2 are sensitive to the toxic effect of METH. At the non-toxic concentration of 1 µM, METH does not affect cell proliferation as assessed by the BrdU incorporation assay. However, the number of

NeuN-positive neurons decreases, as well as P-JNK-dependent axonogenesis in cultures treated with 1 μM METH for 7 days and 6 hours, respectively.

In conclusion, our results show that METH is toxic to SVZ cells and reduces neuronal differentiation and maturation at non toxic concentrations.

Resumo

A metanfetamina é um psicoestimulante muito tóxico e viciante consumido no mundo inteiro, sendo a segunda droga mais consumida a seguir à *cannabis*. Os consumidores de metanfetamina demonstram alterações significativas na função e na estrutura do cérebro. Além disso, o défice de memória frequentemente observado nos consumidores de metanfetamina sugere que, para além do seu efeito tóxico, a metanfetamina poderá alterar a neurogénese. A produção de novos neurónios ocorre no cérebro adulto de mamíferos, incluindo nos humanos. No hipocampo, a neurogénese está associada à capacidade de memorização. Estudos comprovam que em animais injectados com metanfetamina, a proliferação e a génese de células granulares no giro dentado do hipocampo diminuem, o que provavelmente contribui para o decréscimo da memória observado nos consumidores de metanfetamina.

A zona subventricular (ZSV) é a principal região neurogénica no cérebro dos mamíferos e contém células estaminais. Novos neurónios produzidos na ZSV migram através da via rostral migratória e chegam ao bolbo olfactivo. Depois de se diferenciarem em interneurónios, as células recém-chegadas melhoram a função olfactiva. É interessante referir que após haver danificação do cérebro, como em situações de isquémia, epilepsia, trauma cerebral, degeneração de neurónios ou células da glia, a proliferação aumenta na ZSV. As células recém-nascidas abandonam a ZVS e migram em direcção ao local lesado. A ZSV representa a maior fonte de células com potencial reparador no cérebro adulto. Contudo, o conhecimento acerca dos efeitos da metanfetamina na neurogénese e dinâmica das células estaminais da ZSV é reduzido.

Este trabalho foi realizado com o propósito de estudar os efeitos da metanfetamina na neurogénese em culturas celulares da ZSV. Para tal, culturas de neurosferas da ZSV foram obtidas a partir de ratinhos recém-nascidos e expostas a doses crescentes de metanfetamina em condições de suspensão ou aderentes a lamelas durante 48 horas. Desta forma, observámos que a metanfetamina induz morte celular por necrose e apoptose em culturas tratadas durante 24 horas, para concentrações iguais ou maiores que 100 μM. Além disso, demonstrou-se ainda que a metanfetamina não afecta preferencialmente os neuroblastos. No entanto, as células estaminais ou

progenitoras que expressam o factor de transcrição SOX2 são sensíveis ao efeito tóxico da metanfetamina. Relativamente à proliferação, avaliada pelo ensaio de incorporação de BrdU, observámos que a concentrações não tóxicas de 1 μM a metanfetamina não exerce qualquer efeito. Porém, o número de neurónios marcados com NeuN diminui, assim como a axonogénese dependente da fosforilação da JNK em culturas expostas a 1 μM de metanfetamina durante 7 dias e 6 horas, respectivamente.

Em conclusão, os resultados obtidos demonstram que a metanfetamina é tóxica para as células da ZSV e reduz a diferenciação e maturação neuronial a concentrações não tóxicas.

CHAPTER 1

Introduction

1.1. Methamphetamine as a highly toxic drug of abuse

Methamphetamine (METH) is a potent addictive psychostimulant that has dramatic effects on the central nervous system (CNS), and widely abused for its ability to increase wakefulness and physical activity as well as to decrease appetite (Fleckenstein et al., 2007). According to the World Health Organization (http://www.who.int/en), the abuse of amphetamines has become an international public health problem with an estimated 35 million users worldwide, a total which exceeds the number of people who abuse heroin and cocaine. Indeed, methamphetamine constitutes the second most widely abused drug after cannabis (Hamamoto et al., 2009). Importantly, it is estimated that methamphetamine consumption is around 16 million people prevailing upon amphetamine use (United Nations 2007 World Drug Report; Fig. 1.1).

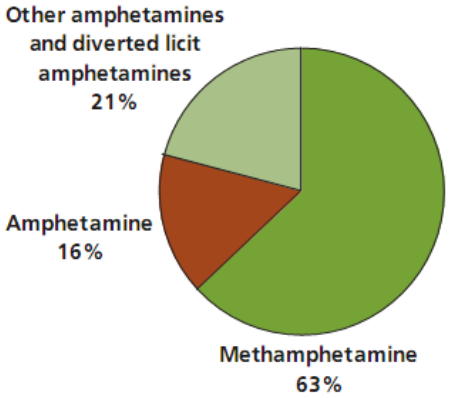


Figure 1.1. Prevalence of methamphetamine, amphetamines and other amphetamines and diverted licit amphetamines use worldwide. (Adapted from the United Nations 2007 World Drug Report.)

In addition, amphetamines consumption affected 1.3% of the young adult Portuguese people in 2007, which was increased comparing to 2001 (Annual Report of the Portuguese Institute for Drugs and Dependence, 2007; Fig. 1.2).

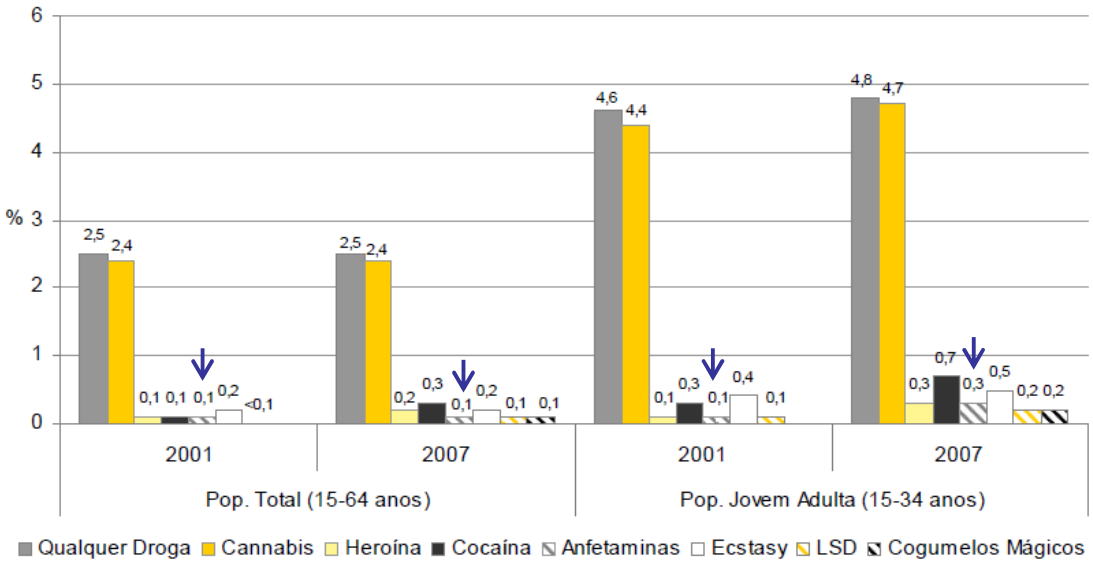


Figure 1.2. Prevalence of drug use in Portugal of total (right) and young adult (left) populations. Amphetamines consumption is indicated with an arrow. (Adapted from the Annual Report of the Portuguese Institute for Drugs and Dependence, 2007; Balsa et al., 2008.)

It has been shown that METH induces neuronal degeneration, apoptosis and neuroinflammation in the brains of human abusers (Zhu et al., 2005; Sekine et al., 2008), which is traduced in several abnormalities in brain function and structure observed in METH abusers (Salo et al., 2009). Additionally, cognitive impairments have also been observed in METH abusers on tasks that require the suppression of task-irrelevant information (Salo et al., 2007), decision making (Paulus et al., 2003) and working memory (McKetin et al., 1998).

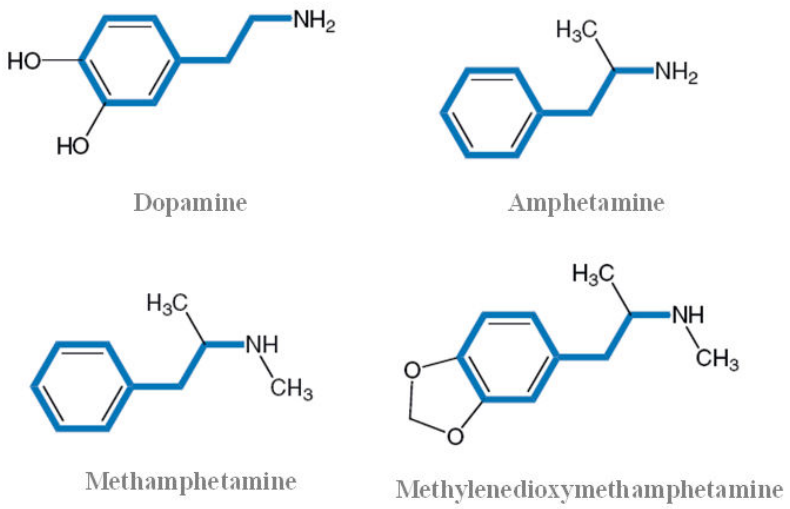


Figure 1.3. Molecular structures of dopamine, amphetamine, methamphetamine and methylenedioxymethamphetamine (MDMA). The three amphetamines share structural features with the neurotransmitter dopamine, consisting of a phenyl ring, a two-carbon side chain (represented in blue) and an amino group bound to carbon-2. METH differs from amphetamine by an additional methyl group connected to the amine that confers METH a lipophylic character able to cross plasmatic membranes of the cells. (Adapted from Fleckenstein et al., 2007.)

METH is highly toxic to the brain acting through several mechanisms that culminate in cell death. Due to the similarity of its chemical structure to dopamine (DA) (Fig 1.3), METH enters to dopaminergic terminals (Iversen, 2006), inhibiting the reuptake of DA via the vesicular monoamine transporter 2 (VMAT-2) and reverting the DA transporter (DAT) of the plasmatic membrane resulting in the increase DA levels in both the cytoplasm and the synaptic cleft (Sulzer et al., 2005; Fig. 1.4). After its displacement to the cytoplasm by METH, DA rapidly auto-oxidizes to form potentially toxic substances including superoxide radicals, hydroxyl radicals, hydrogen peroxide and DA quinones (for review, see Krasnova and Cadet, 2009). These toxic compounds lead to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that lead to an increase in the levels of oxidative stress inside the cell (Sayre et al., 2008). Indeed ROS formation and associated oxidative stress may be involved in METH-related neuronal apoptosis with upregulation of death cascades (Deng and Cadet, 2000).

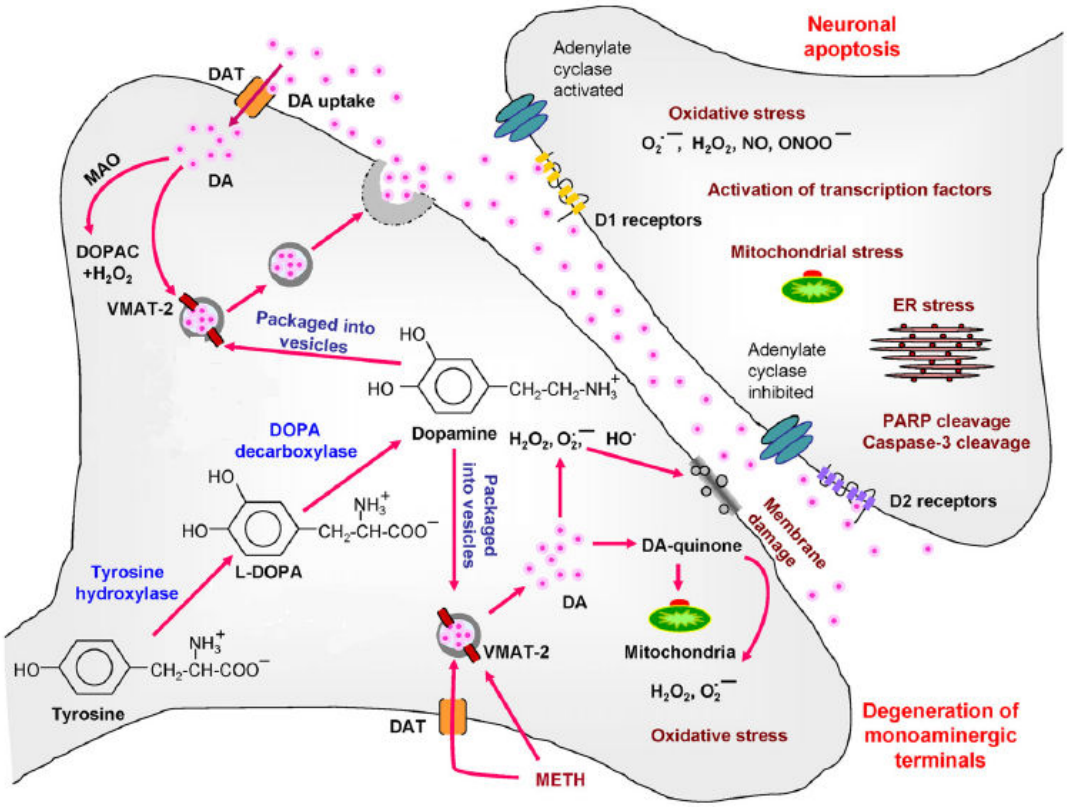


Figure 1.4. Scheme of the cellular and molecular events involved in DA terminal degeneration and neuronal apoptosis induced by METH. (Adapted from Krasnova and Cadet, 2009.)

Glutamate (GLU) is the major excitatory neurotransmitter in the brain and is also a key player of adult neurogenesis. METH increases glutamate levels in the mammalian brain, leading to the hyperactivation of ionotropic receptors, such as *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Consequently, there is an increase in the levels of intracellular calcium and activation of nitric oxide synthase (NOS) which induces the formation of RNS and ROS present in the cytoplasm (Hendrickson et al., 2006; Cadet et al., 2007), leading non-exhaustively to DNA, protein and lipid oxidation and cytoskeletal damage (Davidson et al., 2001; Quinton et al., 2006). Moreover, since METH is a small and lipophylic molecule, it enters directly the cell acting on several organelles. Some evidences suggest that METH leads to an increase in the intracellular calcium concentrations which, in turn, alters the mitochondrial permeability transition pore (PTP) and results in mitochondria disruption, depletions in ATP levels and necrosis

(Davidson et al., 2001). Moreover, when METH enters the mitochondria, the proton gradient is disrupted triggering apoptosis (Davidson et al., 2001). Some accumulated evidence also indicates that METH causes oxidative stress by switching the balance between ROS production and the capacity of antioxidant enzyme systems to scavenge ROS (Krasnova and Cadet, 2009)

Overall, the neurotoxic effects of METH may contribute to the cognitive deficits adjacent to the drug use. Indeed, METH induces apoptosis of pyramidal and interneurons in the cortex, dopaminergic and serotoninergic neurons as well as of hippocampal projection neurons of the CA1 and CA3 regions. Consistently, loss of gray matter is observed in METH abusers. However, neuronal death may not be the only outcome of METH intake. Indeed, the observation of learning and memory impairments in METH-intoxicated patients suggests a deleterious effect of this drug onto hippocampal neurogenesis (Thompson et al., 2004).

1.2. Neurogenesis in the adult brain: focus on the subventricular zone

For almost one century the brain was considered immutable, it was believed that no new neurons were generated in adult brain to replace the dead ones. However in 1969, Joseph Altman showed for the first time that cells in the olfactory bulb, the subventricular zone (SVZ) and in the hippocampus incorporated radioactive thymidine, an indicator of cell proliferation. These cells were identified as being glial cells in the rat and primate brain (Privat and Leblond, 1972; Rakic, 1985). Later, neurogenesis was identified in the adult songbird brain: new neurons are born in the ventricular zone and incorporate into neuronal circuits of the high vocal centre, a telencephalic nucleus involved in song elaboration (Alvarez-Buylla et al., 1992). Evidence of mammalian neurogenesis occurred in 1992, where Reynolds and Weiss demonstrated that the SVZ of mouse contains a population of self-renewing and multipotent cells, displaying the cardinal features of stem cells described by Hall and Watt in 1989.

Neurogenesis begins in the embryo, continues postnatally and into adult life and it can be defined as the process by which a population of neural stem cells generates new neurons. In the adult brain, neurogenesis is a process central to the generation and integration of new neurons into pre-existing neural circuitry, and is crucial for the

maintenance of brain integrity, plasticity and optimal function (Ming and Song, 2005) The genesis of new neurons occurs in the adult mammalian brain in two restricted areas: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone (SVZ) located in the walls of the lateral ventricles. Both niches contain a population of multipotent and self-renewing neural stem/progenitor cells.

1.2.1. Neurogenesis in the dentate gyrus

In the DG, new neurons emerge from stem-like cells residing in the SGZ of the hippocampus, lying between the granule cell layer (GCL) and the hilus. In the SGZ, stem-like cells, identified as astrocytes (B cells), give rise to precursors (D cells) that rapidly divide and differentiate into immature granule cells (type G cells) (Doetsch, 2003). New granule cells migrate locally to the GCL, achieve their maturation and functionally integrate into the pre-existing circuits, receiving inputs from the entorhinal cortex and sending outputs to the CA3 and hilus regions (Zhao et al., 2008; Fig. 1.5). Neurogenesis in the hippocampus is known to be correlated with learning and memory. Accordingly in rodents, stimulation of hippocampal neurogenesis through environmental enrichment and voluntary running increases performances in learning and memory tasks while pharmaceutical suppression of hippocampal neurogenesis impairs the capacity of learning new hippocampus-dependent tasks (for review see Zhao et al., 2008).

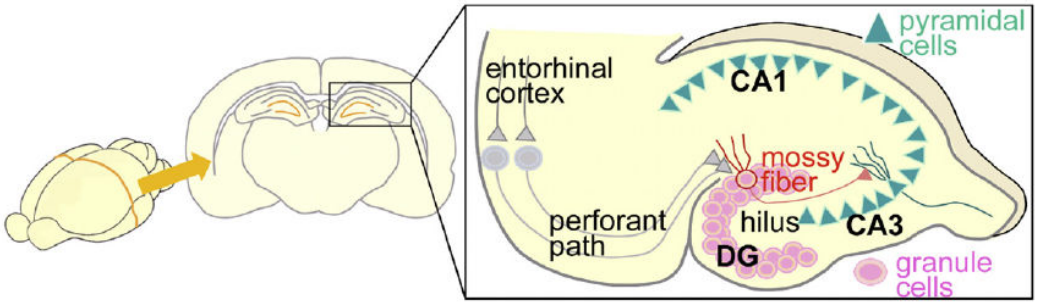


Figure 1.5. Scheme of a coronal section of the adult mouse brain showing the SGZ in the dentate gyrus of the hippocampus. In the SGZ, progenitor cells give rise to granule cells which integrate in existing circuits contributing to learning and memory. (Adapted from Kaneko et al., 2009.)

1.2.2. Neurogenesis in the SVZ

The SVZ is located throughout the lateral wall of the lateral ventricle (Fig. 1.6) and harbours the largest population of proliferating cells in the adult brain of rodents (Gotz and Huttner, 2005), non-human primates (Gould et al., 1999; Pencea et al., 2001) and humans (Bédard and Parent, 2004; Curtis et al., 2007; Sanai et al., 2004 and 2007). In the rodent, adult neurogenesis begins with the proliferation of SVZ neural stem cells, type B astrocytes, which can give rise to fast-cycling transiently proliferating precursor cells that are called type C precursors or progenitor cells. Type C cells, in turn, start to differentiate into type A neuroblasts that are immature neurons mitotically active. These neuroblasts migrate tangentially along the rostral migratory stream (RMS) up to the olfactory bulb (OB) where they drift radially to complete their maturation into neurons (Lois and Alvarez-Buylla, 1994; Wichterle et al., 2001). After maturation, neurons are able to integrate circuits in the olfactory bulb and form synapses (Lledo et al., 2006; Zhao et al., 2008). The new cells have morphological characteristics of granule and periglomerular cells and are important for odour discrimination (Doetsch et al., 1999).

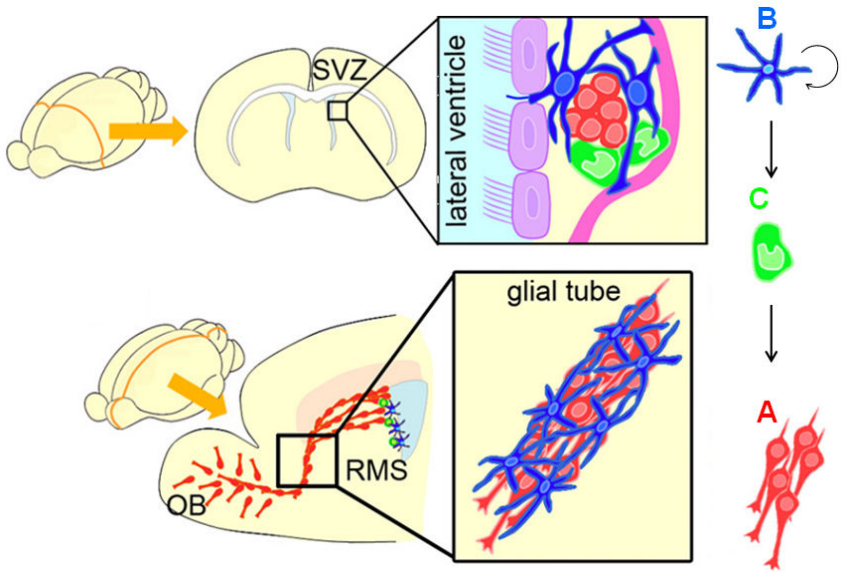


Figure 1.6. Scheme of coronal and sagittal section of the adult mouse brain showing the SVZ adjacent to the lateral ventricle. A subpopulation of SVZ astrocytes (B, blue) displays stem cell properties and generate rapidly dividing transit-amplifying cells (C, green), which differentiate into migrating neuroblasts (A, red) destined for the olfactory bulb. Neuroblasts migrate in chains travelling through tunnels formed by processes of SVZ astrocytes that constitute the rostral migratory stream (RMS). A specialized basal lamina contacts all cell types. (Adapted from Kaneko et al., 2009.)

1.2.3. Notion of neurogenic niches

In the SVZ and the SGZ, the microenvironment is critical for neural stem/progenitor maintenance and neurogenesis. Indeed, proliferation, differentiation, survival and migration of the stem/progenitor cells and their progeny are tightly regulated by diffusible factors, cell-to-cell contact including contacts with the basal lamina and the blood vessels (Alvarez-Buylla and Lim, 2004; Fuchs et al., 2004; Shen et al., 2008; for review see Riquelme et al., 2008). The neurogenic competence of the niches relies on the capacity of grafting neuronal progenitors into these regions to develop into fully mature neurons while they do not in other brain regions (Lois and Alvarez-Buylla, 1994; Ortega-Perez et al., 2007).

1.2.4. SVZ and brain repair

Neurodegenerative disorders are generally characterized by the loss of neurons from specific regions of the brain. Moreover, some data in the literature demonstrate that neurogenesis is altered in the damaged brain (Abdipranoto et al., 2008). Namely, upon injury proliferation increases in the SVZ and newborn cells migrate out of the SVZ towards lesioned areas. In fact, when the brain is subjected to a local aggression, as in striatal ischemic stroke, progenitor cells are recruited from the SVZ and differentiate into neuroblasts that migrate to the boundary of the ischemic lesion, replacing dead striatal spiny neurons (Arvidsson et al., 2002; Jin et al., 2003 and 2006; Zhang et al., 2008). Furthermore, it has been observed that following cortical injury, proliferation and expression of immature neuronal markers are increased in the SVZ and neuroblasts migrate out of the SVZ, reaching the damaged cortical territories (Faiz et al., 2008). Neurogenesis is also increased in the SVZ of patients suffering from Huntington's disease (Curtis et al., 2003) as well as in the hippocampus of Alzheimer's disease patients (Jin et al., 2004). Besides, it has been observed in the brain of patients suffering from multiple sclerosis that repairing potential is acquired through the production of myelinating SVZ-derived oligodendrocytes for the corpus callosum (Nait-Oumesmar et al., 2007). Overall, the accumulated data demonstrate the importance of the SVZ as a source of cell for brain repair.

1.3. Methamphetamine and neurogenesis

Drugs of abuse have been shown to affect adult hippocampal neurogenesis. Accordingly, morphine decreases cell proliferation in the DG (Eisch et al., 2000) while cannabis exert the opposite in cultures of adult progenitor cells (Jiang et al., 2005). Moreover, it was demonstrated that self-administration of nicotine in rats decreases cell proliferation in the SGZ (Abrous et al., 2002).

Concerning the psychostimulants, there is a relative consensus on the fact that these drugs of abuse decrease SGZ and SVZ cell proliferation (Teuchert-Noodt et al., 2000; Yamagushi et al., 2004; Maeda et al., 2007; Noonan et al., 2008). However proliferation is restored to basal levels both in the SVZ and the SGZ following withdrawal from cocaine administration (Noonan et al., 2008). In 2008, Mandyam and colleagues found that intermittent (occasional access) and daily (limited and extended access) self-administration of METH have an impact on different aspects of neurogenesis, the former producing initial pro-proliferative effects and the latter producing downregulating effects. Regarding toxicity, METH was shown to induce cell death in progenitor cells cultures from embryonic rat hippocampus (Tian et al., 2009). Deng and co-workers (2007) found that periglomerular dopaminergic neurons generated in the olfactory bulb of mice are killed by METH altering olfactory processing (Deng et al., 2007). It is noteworthy that few studies address the effect of METH on neurogenesis. Among these studies, attention is mainly focused onto proliferation and little is known about the effect of METH onto stem/progenitor toxicity and capacity of neuronal differentiation.

1.4. Objectives

Therefore, the aim of the present work was to study the effects of METH on SVZ neurogenesis. Despite the fact that olfactory neurogenesis does not occur in humans, human SVZ cells keep neurogenic properties *in vitro* (Sanai et al., 2004 and 2007). For that reason, as being a promising source of repairing cells, the effects of METH on SVZ neurogenesis deserve to be examined.

We treated SVZ cultures derived form early postnatal mice brains with different concentrations of METH. Overall, we evaluated the effect of METH on (1) cell death: as METH neurotoxicity has been reported, METH may trigger cell death in the SVZ cultures both by apoptosis and/or necrosis; (2) cell proliferation: data of the literature point to a inhibitory effect of METH on proliferation, thus proliferation in SVZ cells challenged by METH might decrease; (3) neuronal differentiation; and (4) axonogenesis: impaired neuronal functions in METH abusers suggest alteration in the processes of neuronal differentiation and maturation.

CHAPTER 2

Materials and Methods

2.1. Animal experimental procedures

Experiments were performed in accordance with European Union (86/609/EEC) guidelines for the care and use of laboratory animals. All efforts were made to minimize and to reduce the numbers of animals used.

2.2. Subventricular zone cell cultures

SVZ cells were cultured from 0- to 3-day-old C57Bl/6 donor mice. Animals were sacrificed by decapitation, brains were removed and put in Hanks' balanced saline solution (HBSS, Gibco®, Rockville, MD, http://www.invitrogen.com) during the procedures. Fragments of SVZ were dissected out from 450-µm-thick coronal brain sections, digested in 0.025% trypsin and 0.265 mM EDTA (Gibco) in HBSS solution, followed by mechanical dissociation with a P1000 pipette. The cell suspension was diluted in serum-free culture medium (SFM) composed of Dulbecco's modified Eagle's medium/Ham's F-12 medium GlutaMAX-I (Gibco) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% B27, 10 ng/ml epidermal growth factor, and 5 ng/ml fibroblast growth factor-2 (all from Gibco). Single cells (Fig. 2.1A) were then plated on uncoated Petri dishes at a density of 3 000 cells per cm^2. The neurospheres were allowed to develop in a 95% air-5% CO_2 humidified atmosphere at 37°C. Six- to 8-day-old neurospheres (Fig. 2.1B) were adhered for 48 hours onto poly-D-lysine-coated glass coverslips in SFM devoid of growth factors. Then, the neurospheres were allowed to develop for 6, 24, 48 hours or 7 days at 37°C in the absence or in the presence of 1 µM, 10 µM, 100 µM, 250 µM or 500 µM methamphetamine ((+)-

Methamphetamine hydrochloride, $C_{10}H_{15}N{\cdot}HCl$, from Sigma-Aldrich®, St. Louis, MO), as depicted in the figure 2.2.

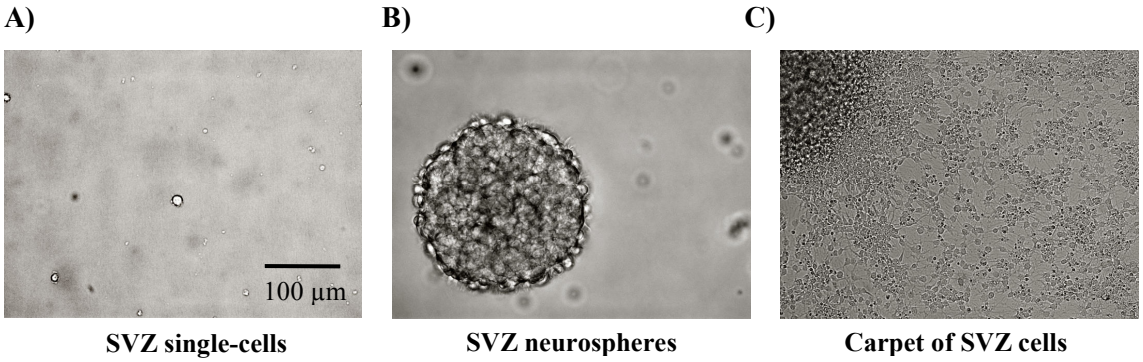


Figure 2.1. Transmission photos of SVZ cell cultures. (A) SVZ neurospheres were obtained from the dissociation of 0- to 3-day-old C57BL/6 mice SVZ explants into single cells and allowed to grow in serum free medium (SFM) in the presence of EGF and FGF-2. **(B)** 6- to 8-day-old neurospheres were plated onto poly-D-lysine coated coverslips and incubated with SFM devoid of growth factors. **(C)** 48 hours of incubation in differentiating conditions provided the formation of a carpet of differentiated cells where the analyses were performed.

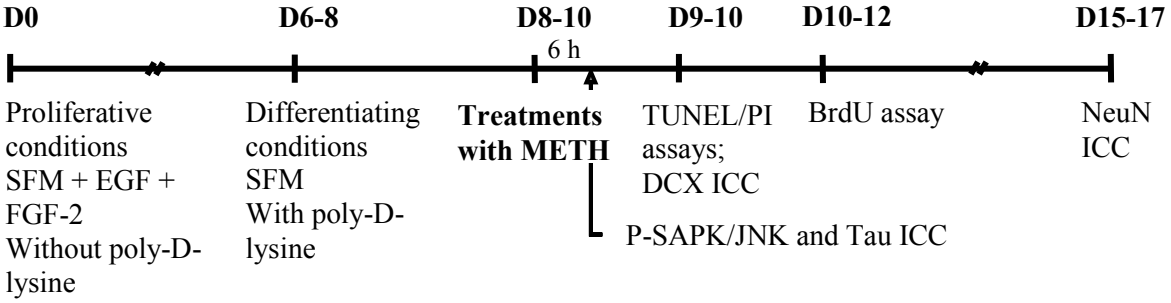


Figure 2.2. Scheme for general protocol. SFM, serum-free culture medium; ICC, immunocytochemistry.

2.3. Immunocytochemistry

After fixation for 30 minutes in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature (RT), cells were permeabilized and non-specific binding sites were blocked for 1 hour and 30 minutes with 0.25% Triton X-100 (Sigma-Aldrich) and 6% bovine serum albumin (BSA, Sigma-Aldrich) dissolved in PBS. Cells were then subsequently incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-neuronal nuclear protein (anti-NeuN, 1:100; Chemicon®, Temecula, CA, http://www.chemicon.com), rabbit polyclonal anti-doublecortin (anti-DCX, 1:200), mouse monoclonal anti-Tau (1:500), rabbit polyclonal anti-phosphorylated form of stress-activated protein kinase/c-Jun-NH_2-terminal kinase (anti-P-SAPK/JNK, 1:100; all from Cell Signaling Technology®, Danvers, MA, http://www.cellsignal.com) or goat polyclonal anti-SOX2 (1:100; from Santa Cruz Biotechnology®, Santa Cruz, U.S.A.). Thereafter, coverslips were rinsed in PBS and incubated for 1 hour at RT with the appropriate secondary antibodies as following: anti-rabbit IgG labelled with Alexa Fluor 488 or Alexa Fluor 594 (1:200), anti-mouse IgG labelled with Alexa Fluor 488 or Alexa Fluor 594 (1:200) or anti-goat IgG labelled with Alexa Fluor 594 (1:200; all from Molecular Probes®, Oregon, USA). The list of antibodies used in immunocytochemistry is resumed below in Table I. Nuclei were counterstained with Hoechst 33342 (2 µg/ml in PBS containing 0.25% BSA; Molecular Probes). Preparations were mounted in DakoCytomation® fluorescent medium (DakoCytomation, CA, http://www.dakocytomation.com).

Table I - Antibodies used in immunocytochemistry.

Target	Primary antibody	Dilution	Origin	Secondary antibody	Dilution	Origin
Mature neurons	Mouse monoclonal anti-NeuN (MAB317)	1:100	Cell Signaling Technology, Danvers, MA	Alexa Fluor 488 goat anti-mouse IgG (A11001)	1:200	Molecular Probes, Oregon, USA
Migratting immature neurons	Rabbit polyclonal anti-DCX (4604)	1:200	Cell Signaling Technology	Alexa Fluor 594 goat anti-rabbit IgG (A11012)	1:200	Molecular Probes
Developing and mature neurons	Mouse monoclonal anti-Tau (4019)	1:500	Cell Signaling Technology	Alexa Fluor 594 goat anti-mouse IgG (A11005)	1:200	Molecular Probes
Growing axons	Rabbit polyclonal anti-SAPK/JNK (9251)	1:100	Cell Signaling Technology	Alexa Fluor 488 goat anti-rabbit IgG (A11008)	1:200	Molecular Probes
Stem/ progenitor cells	Goat polyclonal anti-SOX2 (sc-17320)	1:100	Santa Cruz Biotechnology, Santa Cruz, U.S.A.	Alexa Fluor 594 rabbit anti-goat IgG (A11080)	1:200	Molecular Probes
Dividing cells	Anti-BrdU monoclonal antibody Alexa Fluor 594 conjugate (A21304)				1:100	Molecular Probes

BrdU, 5-bromo-2'-deoxyuridine; **DCX**, doublecortin; **P-SAPK/JNK**, phosphorylated form of stress-activated protein kinase/c-Jun-NH_2-terminal kinase; **NeuN**, neuronal nuclear protein.
For more details see http://www.cellsignal.com, http://www.invitrogen.com, http://www.scbt.com.

2.4. Neuronal cell death assay

Both cell death by necrosis and apoptosis were detected. The number of necrotic cells was measured using the propidium iodide assay (PI, 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide; from Sigma-Aldrich). PI is a fluorescent dye that enters the cells when plasmatic membrane is damaged, which when exposed to blue-green light (493 nm) emits a bright red fluorescence (630 nm). Living cells were incubated with 3 µg/µl PI for the last 40 minutes of the culture session that had a total duration of 24 hours (Fig. 2.2). Cells were fixed in 4% PFA in PBS. Thereafter, the terminal deoxynucleotidyl transferase dUTP

nick-end labelling (TUNEL) method was performed to detect apoptotic nuclei. The TUNEL method is based on the activity of the enzyme terminal transferase which attaches labelled nucleotides (biotin-dUTP) to the 3'-OH ends of the DNA generated during apoptotic-induced DNA fragmentation. Briefly, cells were permeabillized in 0.25% Triton-X-100 and subsequently incubated in terminal deoxynucleotidyl transferase buffer (pH 7.5) containing terminal transferase (0.25 U/µl) and biotinylated dUTP (6 µM) (all from Roche Diagnosis®, Mannheim, Germany) for 1 hour at 37°C. Enzymatic reaction was stopped by incubating the cells in TB buffer (300 mM NaCl: 30 mM sodium citrate). The biotinylated dUTPs were revealed by fixation of Fluorescein Avidin D (1:100; from Vector Laboratories®, Burlingame, CA) for 30 minutes. Nuclei counterstaining and mounting were performed as described previously. Moreover, in order to confirm the pro-apoptotic effect of METH observed by the TUNEL assay, cells were incubated with a specific general caspase, z-Val-Ala-DL-Asp (OMe)-fluoromethylketone (ZVAD, 20 mM; Calbiochem®, California, USA). ZVAD was diluted in 1:800 DMSO to obtain a final concentration of 25 µM.

2.5. Progenitor cell death assay

Quantification of progenitor cells death was performed in 4-5 day-old neurospheres, treated for the last 24 hours of the culture session in the absence (control) or the presence of 250 µM of METH, dissociated using a P1000 pipette and adhered to SuperFrost® Plus glass slides (Thermo Scientific®, Menzel GmbH & Co KG®, Braunscheweig, Germany; http://www.menzel.de) by centrifugation (425g/2000 rpm, 5 min; Cellspin I®, Tharmac GmbH®, Waldsoms, Germany; http://www.tharmac.de). Cells were then fixed with 4% PFA in PBS for 30 minutes, and further labelled with the transcription factor SOX2. Subsequently, TUNEL assay was performed and nuclei counterstaining was performed as described previously. The treatments applied to the cells are summarized in the following timeline (Fig. 2.3).

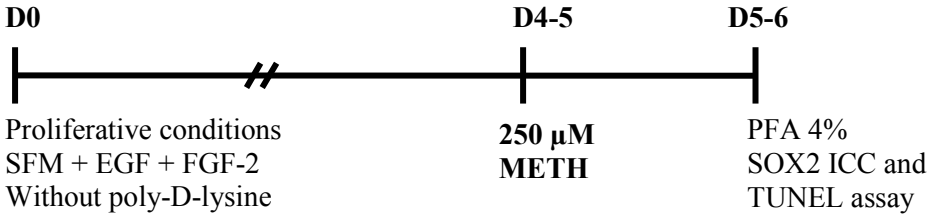


Figure 2.3. Scheme for experimental protocol. After dissociation of SVZ explants into single cells, the cells were allowed to grow in serum free medium (SFM) in the presence of EGF and FGF-2 for 4 to 5 days. To evaluate cell death in stem/progenitor cells, treatments were applied for 24 hours on free-floating neurospheres before fixation.

2.6. Proliferation Assay

5-Bromo-2'-deoxyuridine (BrdU, 10 µM; Sigma-Aldrich) was added in the last 4 hours of the culture session that had a total duration of 48 hours (Fig. 2.2). BrdU is a thymidine analogue that is incorporated in the DNA of cells in the S-phase of the cell cycle. BrdU was then unmasked following successive passages in 1% Triton X-100 for 30 minutes, ice-cold 0.1 N HCl for 20 minutes, and finally 2 N HCl for 40 minutes at 37°C. After that, acid was neutralized by incubating the cells with borate buffer (0.1 M $Na_2B_4O_7 \cdot 10H_2O$, pH 8.5) for 15 minutes at RT. Subsequently, coverslips were rinsed in PBS and non-specific binding sites were blocked with 3% BSA (Sigma-Aldrich) and 0.3% Triton X-100 in PBS for 30 minutes at RT. Cells were then incubated with the anti-BrdU monoclonal antibody Alexa Fluor 594 conjugate (1:100; from Molecular Probes) in PBS containing 0.3% BSA (Sigma-Aldrich) and 0.3% Triton-X100 for 1 hour and 30 minutes at RT and left overnight at 4°C. After rinsing the coverslips, nuclei counterstaining and mounting were performed as described previously.

2.7. Data analysis and Statistics

Transmission images were took using a Zeiss®/P.A.L.M. Laser Dissecting Microscope and fluorescent images were recorded using a LSM 510 Meta® confocal microscope or an Axioskop 2 Plus® fluorescent microscope (all from Carl Zeiss®, Göttingen, Deutschland). Except for the progenitor cell death assay for which

measurements were achieved in a monolayer of dissociated cells, measurements were performed at the border of the neurospheres, where migrating cells emerged, forming a dense cell monolayer. Each experimental condition was assayed in two or three different wells. Except where otherwise specified, the experiments were duplicated. Percentages of BrdU- and NeuN-immunoreactive cells, as well as TUNEL and/or PI labelled cells were derived from cells counted in five independent microscopy fields in each coverslip with a ×40 objective (approximately 200 cells per field). Percentages of the numbers of DCX-immunoreactive and TUNEL labelled cells were derived from cells counted in ten independent microscopy fields in each coverslip with a ×40 objective (approximately 200 DCX-immunoreactive cells per coverslip). Percentages of the numbers of SOX2-immunoreactive and TUNEL labelled cells were derived from cells counted in dissociated neurospheres adhered to a microscope slide (in a density of 200 to 300 SOX2-immunoreactive cells per coverslip). Cells were counted in two different slides for each condition from two different cultures. Quantifications of P-SAPK/JNK-positive nuclei at 6 hours were done in two independent cultures in at least 20 non-overlapping fields (magnification, ×400). Measurements of total length (μm) of the ramifications and quantification of the number of ramifications per neurosphere were done in approximately 20 non-overlapping fields in each coverslip using digital images (two coverslips from two different cultures). Percentages of statistical significance were determined using two-tailed Student's *t* test for comparison between two groups and one-way ANOVA analysis of variance for comparison between more than two groups followed by Dunnett's multiple comparison test or Bonferroni's multiple comparison test to compare groups to control or pairs of groups, respectively. All data are presented as means ± SEM (standard error of the mean). Statistical significance level was set for p values < 0.05.

CHAPTER 3

Results

3.1. Methamphetamine exerts toxic effects in SVZ cell cultures

To investigate the effect of METH, single cells obtained from 6-8 day-old primary neurospheres, were incubated in the absence (control) or the presence of 1 μM and 250 μM of METH and secondary neurospheres were allowed to develop for 4 days (Fig. 3.1). At the end of the culture session, round shaped neurospheres presenting a diameter of 100 to 300 μm are found in the control cultures (Fig. 3.2A). METH at 1 μM seemingly has no effect on neurosphere development as cultures display a similar aspect comparing to the control cultures (Fig. 3.2B). However, neurospheres appeared shrunken and in a lesser density in cultures treated with 250 μM METH. Moreover, cellular debris was often observed in the 250 μM METH treated cultures (Fig. 3.2C). These observations allow us to conclude that METH, at 250 μM but not at 1 μM, impairs neurosphere development and cellular viability.

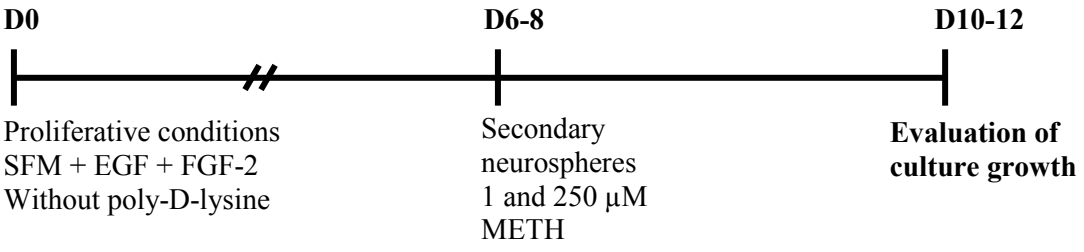


Figure 3.1. Scheme for experimental protocol.

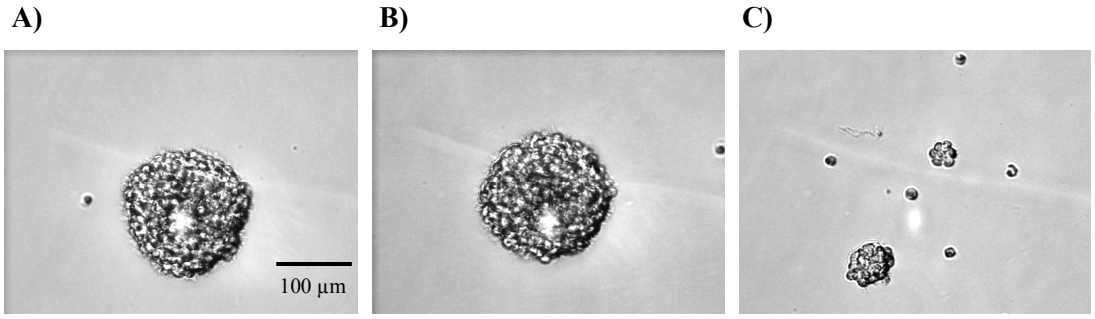


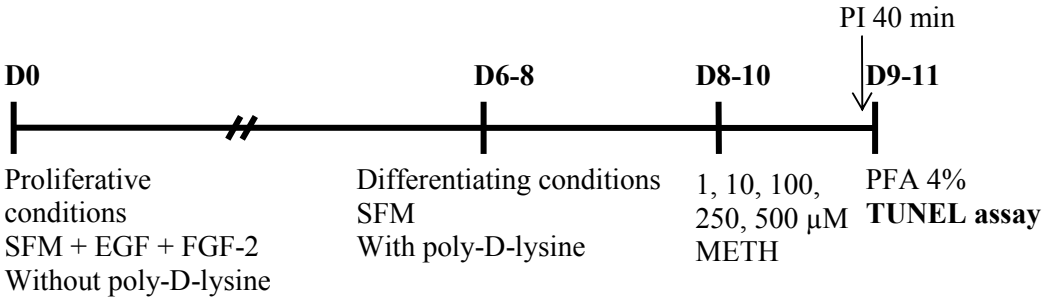
Figure 3.2. Transmission photos for culture growth. (A) Representative transmission photos of SVZ neurospheres cultured in the absence (control) **(B)** or presence of 1 µM **(C)** or 250 µM of methamphetamine.

To disclose whether METH is toxic for SVZ cells, 6-8 day-old neurospheres were adhered for 48 hours on poly-D-lysine coated coverslips and then treated for 24 hours with 1 to 500 µM of METH. Propidium iodide (PI) is added for the last 40 min of the culture session. Cells are then fixed with PFA 4% in PBS and proceeded for TUNEL staining to reveal apoptotic cell nuclei (Fig. 3.3A). As described by Kelly and collaborators (2003), necrotic cells and apoptotic cells can be visualized and discriminated using the TUNEL reaction sequentially to PI uptake assay. During necrosis upon an injury, cells swell and their plasmatic membrane eventually ruptures. PI binds to double-stranded DNA, but it can only enter the cells when plasmatic membrane is damaged, so cells in necrosis or in late apoptosis uptake PI. Apoptosis or programmed cell death relies in the fragmentation of the chromatin. The TUNEL staining labels specifically the apoptotic cells as it consists in the addition of labelled dUTPs to the free DNA ends generated by activated endonucleases during the apoptotic cycle. In this way, apoptotic cells present TUNEL-positive nuclei and in the case of cells being in late apoptosis they are also PI-positive. Confocal photos depict PI (red) and TUNEL (green) staining in control and 250 µM treated cultures (Fig. 3.3B). For clarity, in these figures only some examples of each type of nuclear staining are labelled.

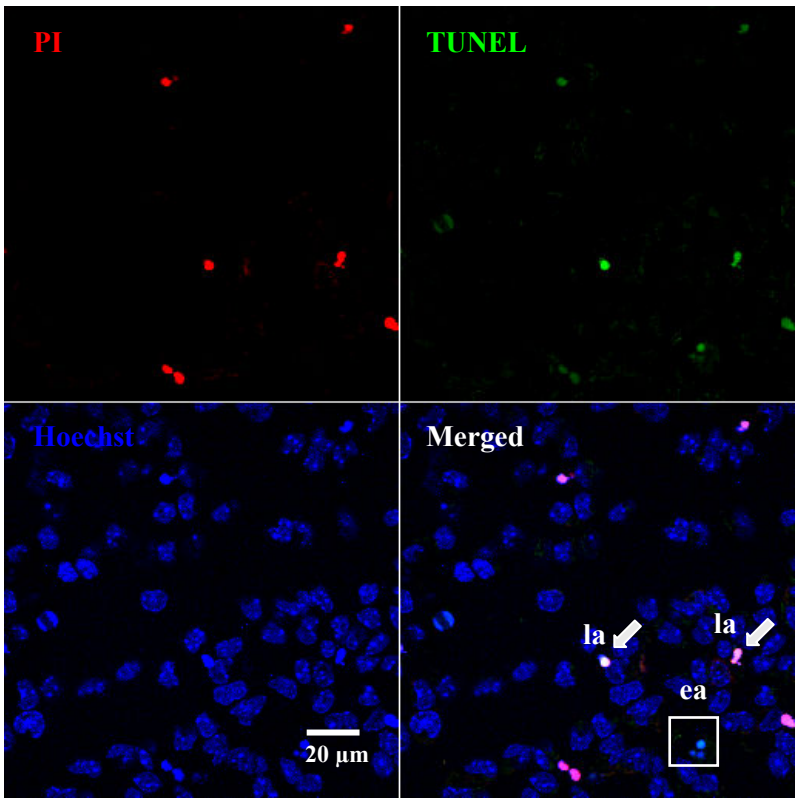
Total cell death is evaluated by counting of both PI- and TUNEL-positive cells and expressed as percentages of total cells stained with Hoechst 3342. As represented in figure 3.3C, METH induces cell death in SVZ cell cultures in a concentration-dependent manner. No effect of METH was observed at a concentration of 1 µM and 10

μM. In control, the level of cell death is around 15% according to basal cell death inherent to SVZ cultures (Control: 14.42 ± 0.37%, METH: 1 μM: 15.48 ± 1.03%, 10 μM: 15.87 ± 0.68%, 100 μM: 19.83 ± 1.68%, 250 μM: 20.93 ± 1.37%, 500 μM: 23.02 ± 1.48%; Fig. 3.3C).

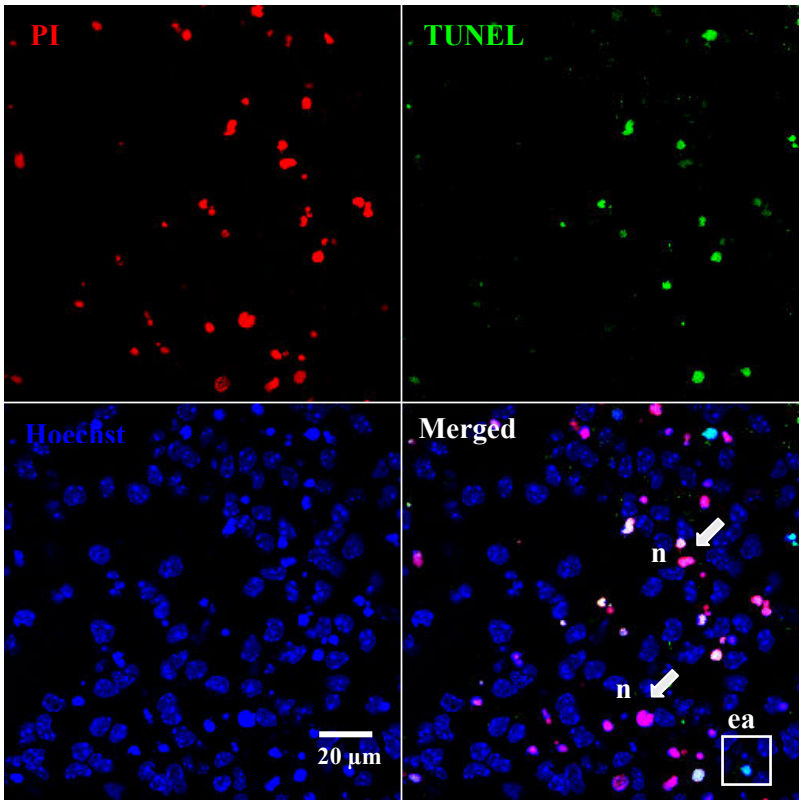
A)



B) Control



250 µM



C)

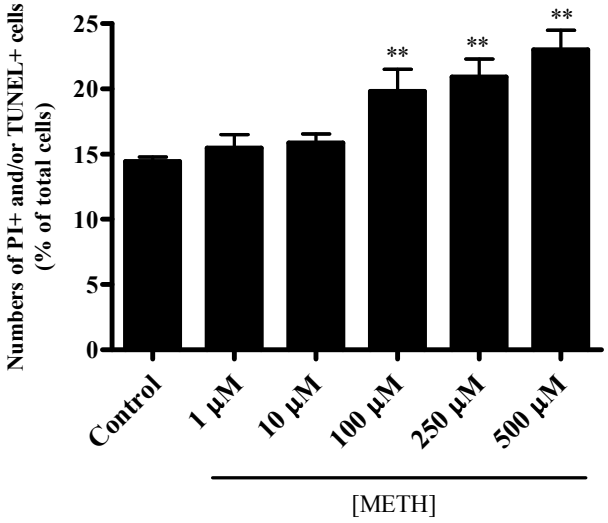


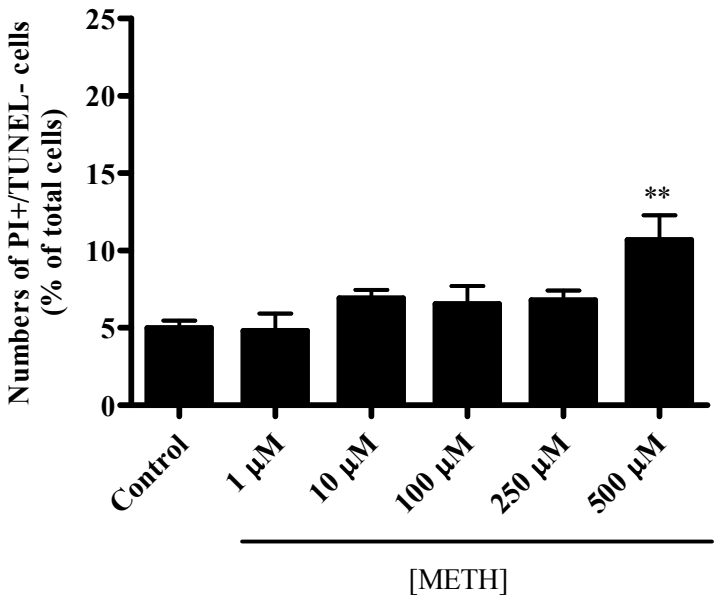
Figure 3.3. Methamphetamine induces cell death in SVZ cell cultures. (A) Scheme for experimental protocol. **(B)** Representative confocal photos of cell nuclei in SVZ cell cultures maintained for 24 hours in the absence (control) or presence of 250 µM METH, labelled for TUNEL (green) and PI (red). **(C)** Bar graph depicts the numbers of dead cells that are PI-positive and/or TUNEL-positive cells, expressed as percentages of the total number of nuclei per culture, in treated and non-treated cultures. Data are expressed as a mean ± SEM (n = 2 independent experiments). ** $p < 0.01$ using one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test for comparison with SVZ control cultures. ea, early apoptosis; la, late apoptosis; n, necrosis. PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling.

Necrosis is induced by METH only at the highest concentration, while at lower concentrations of METH levels of necrosis remained close to control (Control: 4.98 ± 0.50%; METH: 1 µM: 4.80 ± 1.12%, 10 µM: 6.93 ± 0.51%, 100 µM: 6.56 ± 1.13%, 250 µM: 6.81 ± 0.59%, 500 µM: 10.69 ± 1.58%; Fig. 3.4A).

METH triggers apoptosis from 100 to 250 µM, while at the highest concentration no effect on apoptosis was observed (Control: 9.44 ± 0.54%, METH: 1 µM: 10.67 ± 1.45%, 10 µM: 8.94 ± 0.93%, 100 µM: 15.50 ± 1.37%, 250 µM: 14.61 ± 1.40%, 500 µM: 11.65 ± 1.51%; Fig. 3.4B). Activation of caspases is a biochemical hallmark of apoptosis. Caspases are intracellular proteases that propagate programmed cell death (apoptosis) and other biological processes. Studies on substrate specificity, prodomain structure and biological function have revealed that caspases are activated during apoptosis in a self-amplified cascade (Pop and Salvesen, 2009). To confirm that

METH triggers apoptosis in SVZ cell cultures, the specific general caspase inhibitor z-Val-Ala-DL-Asp (OMe)-fluoromethylketone (ZVAD) was added to the control cultures and also to the cultures treated with 250 μM. A decrease in the numbers of TUNEL labelled cells was observed in SVZ cultures treated only with ZVAD, as compared to the control cultures, consistent with the blocking of basal apoptosis. In METH treated cultures, ZVAD prevented the increase in TUNEL-positive nuclei confirming that METH triggers apoptosis (250 μM + ZVAD: 5.74 ± 1.16%, ZVAD: 5.26 ± 0.40%; Fig. 3.4B). Moreover, as ZVAD was diluted in DMSO, as described previously in section 2.4, no toxic effect was induced by DMSO (DMSO: 9.05 ± 0.38% for two independent experiments), as in accordance to the described by Bernardino and collaborators (2008).

A)



B)

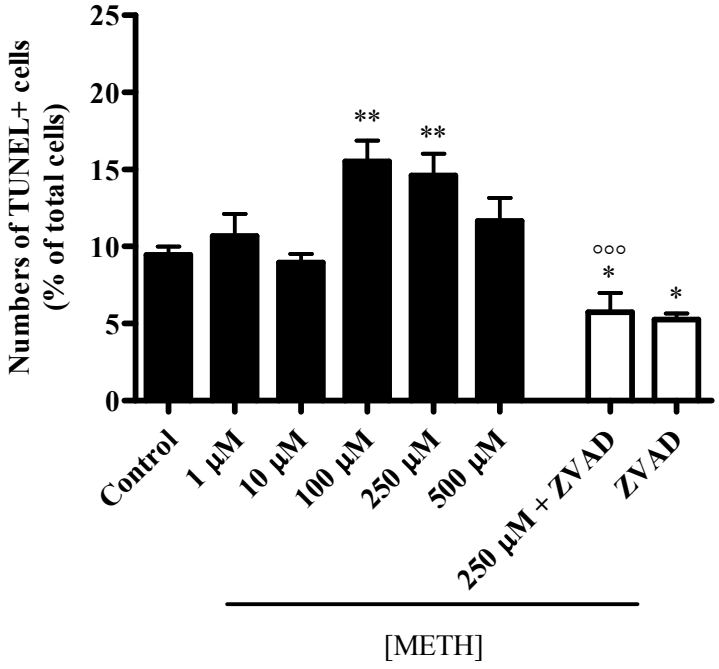


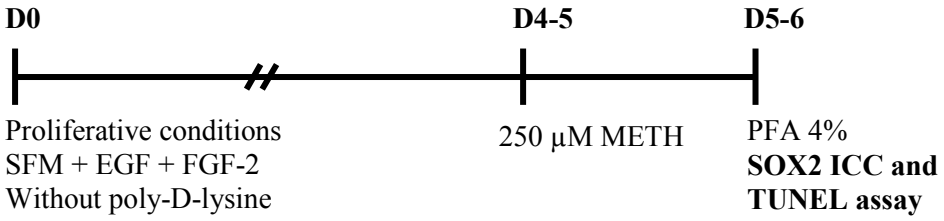
Figure 3.4. (A) METH induces necrosis in SVZ cell cultures. Bar graph depicts the numbers of PI-positive and TUNEL-negative cells, expressed as percentages of the total number of nuclei per culture, in treated and non-treated cultures. **(B) METH triggers apoptosis in SVZ cell cultures, which is prevented by the administration of ZVAD, a specific general caspase inhibitor.** Bar graph depicts the numbers of TUNEL-positive cells, expressed as percentages of the total number of nuclei per culture, in treated and non-treated cultures. Data are expressed as a mean ± SEM (n = 2 independent experiments). * $p < 0.05$, ** $p < 0.01$ using one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test for comparison with SVZ control cultures; °°° $p < 0.001$ using one-way ANOVA analysis of variance followed by Bonferroni's multiple comparison test for comparison with SVZ cultures treated with 250 µM of METH for 24 hours. ZVAD, z-Val-Ala-DL-Asp (OMe)-fluoromethylketone.

3.2. Methamphetamine triggers stem/progenitor cells apoptosis but does not affect preferentially immature neurons

We then determined which cellular populations were targeted by METH-induced toxicity. To investigate whether METH triggers apoptosis in stem/progenitor cells, 4-5 day-old neurospheres were treated for 24 hours with 250 µM METH, dissociated into single cells and adhered to microscope slides by centrifugation in a cytospin. After

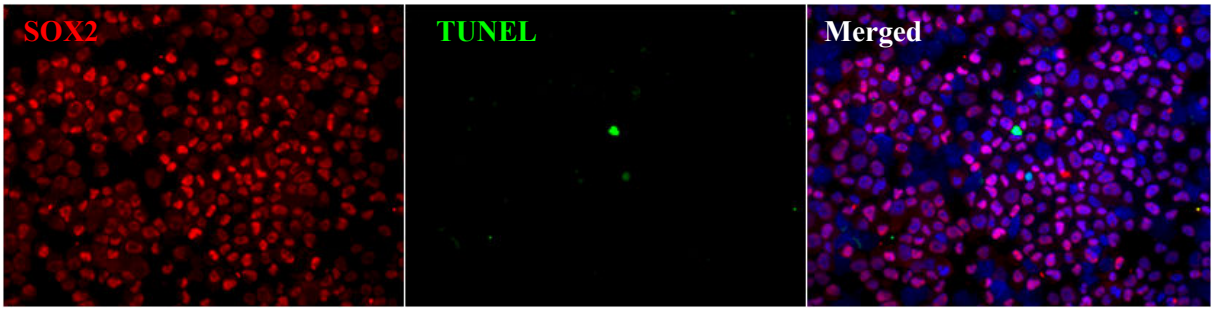
fixation with PFA 4% in PBS, cells were proceeded for SOX2 immunocytochemistry and TUNEL staining (Fig. 3.5A). Fluorescence photos depict SOX2-positive stem/progenitor cells and TUNEL-positive cells in control and METH treated cultures. As expected, more TUNEL-positive cells are present in METH treated cultures (Fig. 3.5B). The total numbers of SOX2- and TUNEL-positive cells were counted and represented as percentages of total SOX2-immunoreactive cells. METH increases the number of SOX2- and TUNEL-positive cells while comparing to the control cultures, indicating that METH is toxic for stem/progenitor cells (Control: 0.41 ± 0.16%, 250 µM METH: 1.78 ± 0.14%; Fig. 3.5C).

A)

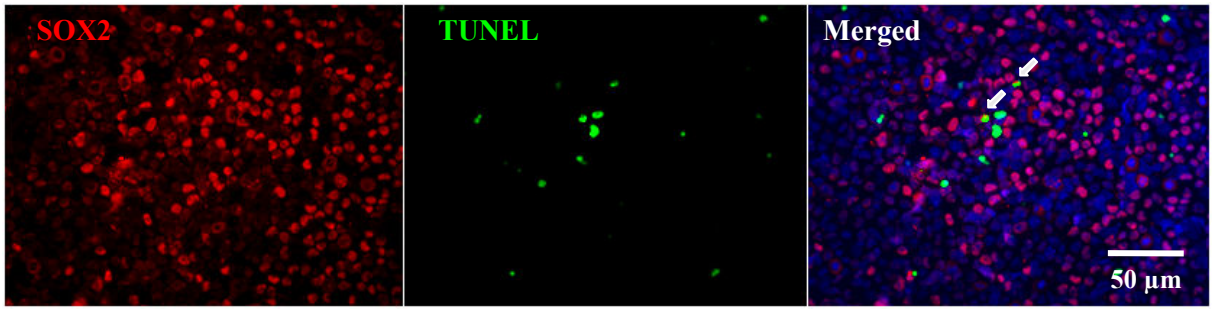


B)

Control



250 µM METH



C)

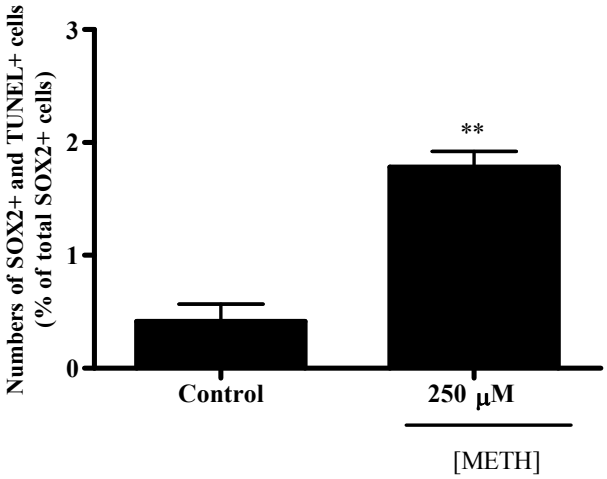
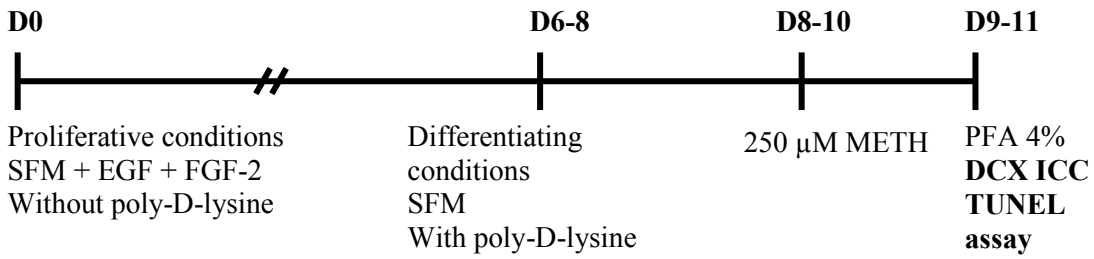


Figure 3.5. METH induces death of stem/progenitor cells in SVZ cell cultures. (A) Scheme for experimental protocol. **(B)** Representative fluorescence photos of SVZ cell nuclei in SVZ cell cultures maintained in suspension for 24 hours in the absence (control) or in the presence of 250 μM METH and labelled for TUNEL (green) and SOX2 (red). Cellular nuclei are stained with Hoechst 33342 (blue). **(C)** Bar graph depicts the numbers of SOX2-positive and TUNEL-positive cells, expressed as percentage of SOX2 labelled cells, in control cultures and in cultures exposed to 250 μM METH for 24 hours. Data are expressed as a mean ± SEM (n = 2 independent experiments). ** $p < 0.01$ using the unpaired student's t test for comparison with SVZ control cultures.

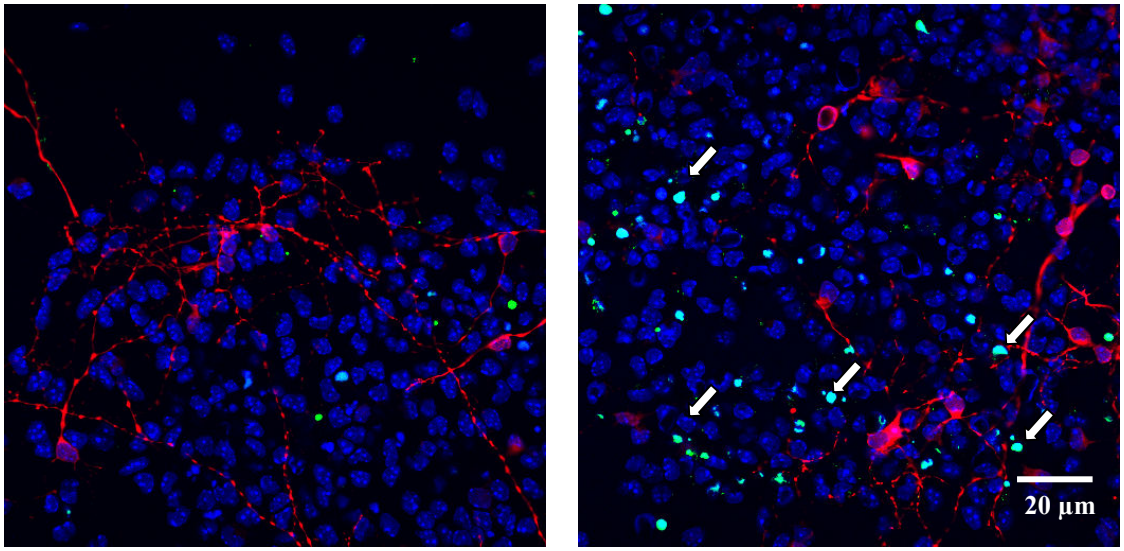
To determine whether the immature neurons were targeted by METH-induced toxic effects, SVZ cell cultures adhered for 48 hours on poly-D-lysine were incubated for 24 hours with 250 μM METH and proceeded for TUNEL staining and doublecortin (DCX) immunolabelling (Fig. 3.6A). DCX is a microtubule-associated protein that is exclusively expressed in post-mitotic neurons during periods of migration, and therefore, migrating immature neurons can be detected using DCX immunofluorescence (Gleeson et al., 1999). Representative photos of the DCX-immunoreactive immature neurons are represented below (Fig. 3.6B-C). The number of DCX- and TUNEL-positive cells were counted and expressed as percentages of total DCX-positive cells. No differences in the numbers of DCX- and TUNEL-positive cells were observed in the cultures, although an increase in total cell death was visible when comparing treated to non-treated cultures (Control: 5.18 ± 0.44%, 250 μM METH: 6.48 ± 0.88%; Fig. 3.6D).

A)

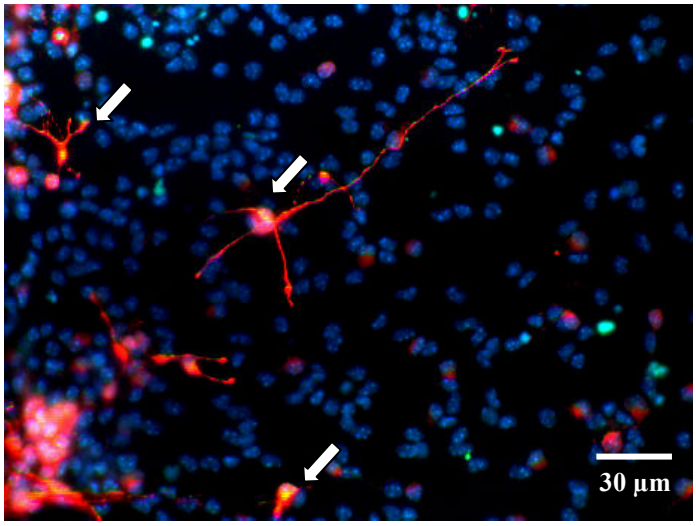


B)

Control **250 µM METH**



C)



D)

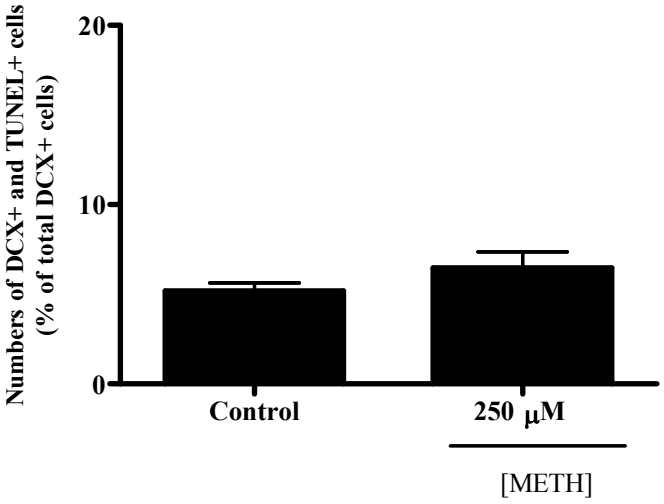


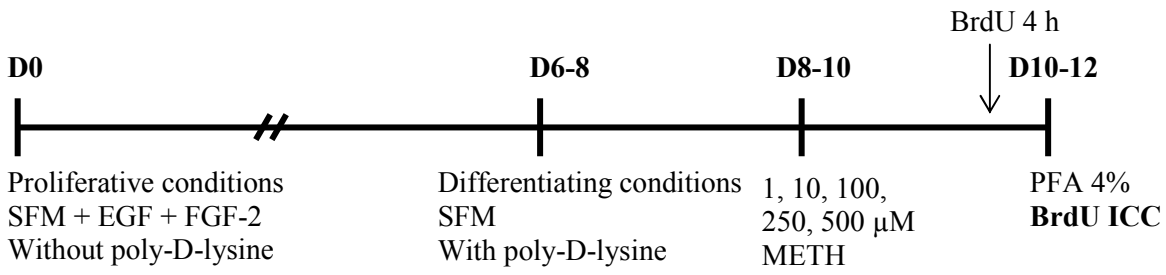
Figure 3.6. METH-induced toxic effects do not preferentially affect immature neurons. (A) Scheme for experimental protocol. **(B)** Representative confocal photos of DCX-stained immature neurons in SVZ cell cultures in the absence (control) or presence of 250 μM METH, labelled for TUNEL (green) and DCX (red). **(C)** Representative fluorescence photo of DCX-stained immature neurons colocalized with TUNEL labelling in SVZ cell cultures treated with 250 μM METH. **(D)** Bar graph depicts the numbers of DCX-positive and TUNEL-positive cells, expressed as percentages of DCX labelled cells, in treated and non-treated cultures. Data are expressed as a mean ± SEM (*n* = 2 independent experiments). DCX, doublecortin.

3.3. Methamphetamine has no effect on cell proliferation

To investigate the effect of METH on cell proliferation 6-8 day-old neurospheres were adhered for 48 hours on poly-D-lysine and treated with METH at a concentration of 1, 10, 100, 250 or 500 μM. For the last 4 hours of the culture session, with a total duration of 48 hours, the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) was added to the cultures (Fig. 3.7A). The numbers of BrdU-immunoreactive cells were counted and expressed as percentages of the total number of nuclei. A decrease in the numbers of BrdU-positive nuclei was observed in the cultures treated with 100, 250 and 500 μM METH. However, since at these concentrations METH was found to be toxic for the cells, as described previously in section 3.1 (Fig. 3.3C), the decrease in BrdU incorporation is rather due to cell death than to an inhibition of proliferation. Additionally, at non-toxic concentrations, no differences in the numbers of BrdU-

positive cells were observed as compared to control, which led to conclude that METH does not affect cell proliferation (Control: 8.63 ± 0.69; METH: 1 µM: 8.10 ± 1.06%, 10 µM: 7.89 ± 0.74%, 100 µM: 5.89 ± 0.33%, 250 µM: 5.18 ± 0.58%, 500 µM: 4.72 ± 0.93%; Fig. 3.7B).

A)



B)

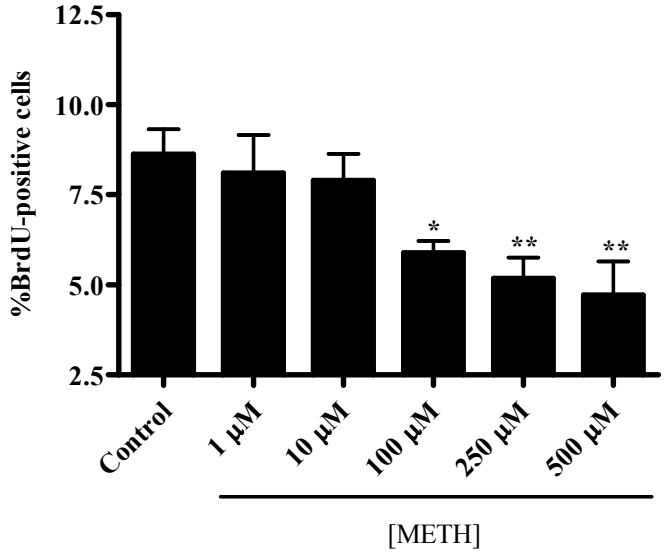
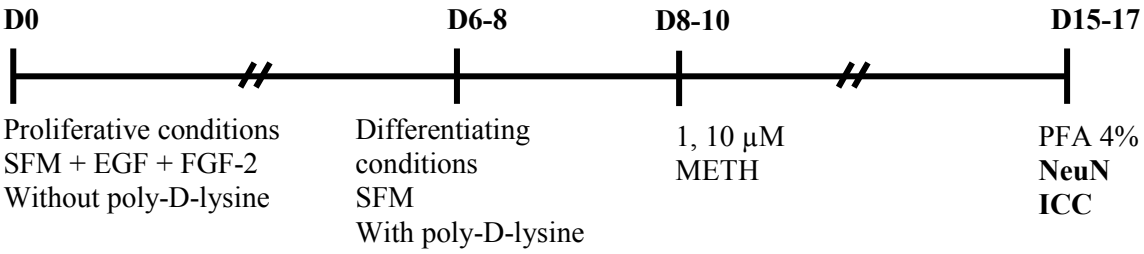


Figure 3.7. METH decreases BrdU incorporation in SVZ cell cultures. **(A)** Scheme for experimental protocols. **(B)** Bar graph depicts the numbers of BrdU-positive, expressed as percentages of total number of nuclei per culture, in treated and non-treated cultures. Data are expressed as a mean ± SEM ($n = 2$ independent experiments). * $p < 0.05$, ** $p < 0.01$ using one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test for comparison with SVZ control cultures. BrdU, 5-bromo-2'-deoxyuridine**.**

3.4. Methamphetamine decreases neuronal differentiation and axonogenesis

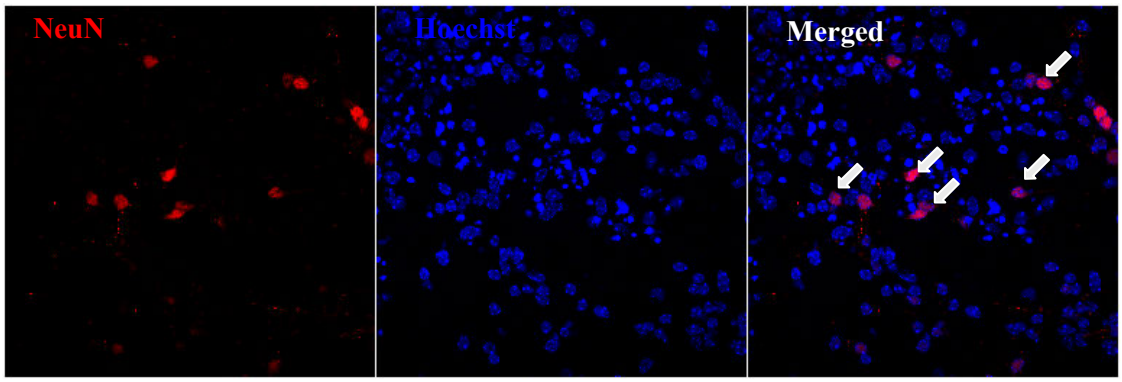
To investigate the effect of METH on neuronal differentiation, SVZ neurospheres adhered for 48 hours onto poly-D-lysine-coated coverslips were incubated for 7 days in the absence (control) or presence of non-toxic concentrations of METH (Fig. 3.8A). At the end of the culture session, mature neurons expressing the nuclear marker NeuN were labelled as illustrated below (Fig. 3.8B). The numbers of NeuN-positive cells were counted and expressed as percentages of total cells. METH at 1 and 10 μM significantly decreases the numbers of NeuN neurons as compared to control cultures, suggesting that METH, at a non-toxic concentration, impairs neuronal differentiation in SVZ cell cultures (Control: 10.72 ± 1.61%, METH: 1 μM: 7.00 ± 0.40%, 10 μM: 6.81 ± 0.75%; Fig. 3.8C).

A)

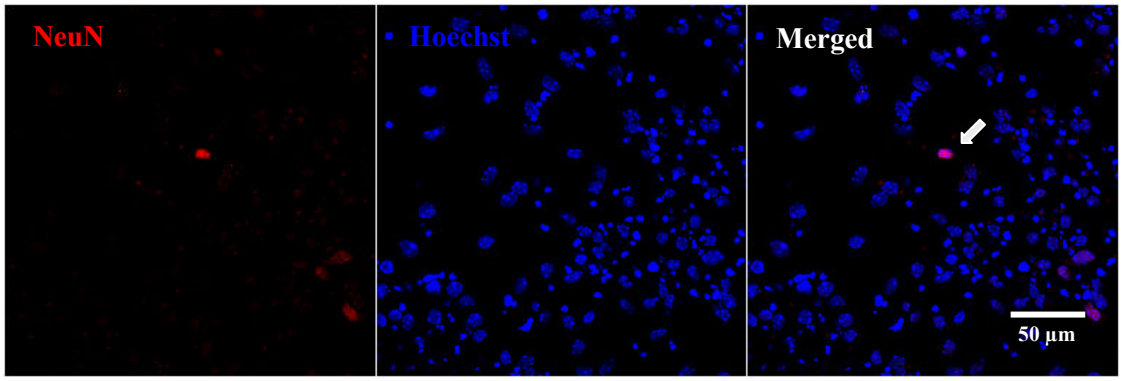


B)

Control



1 µM METH



C)

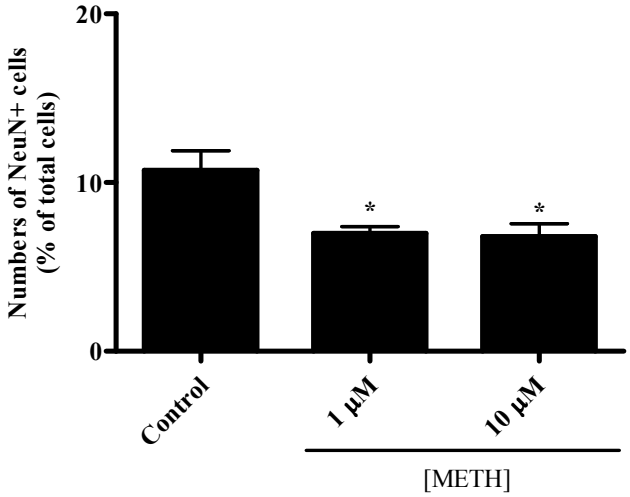


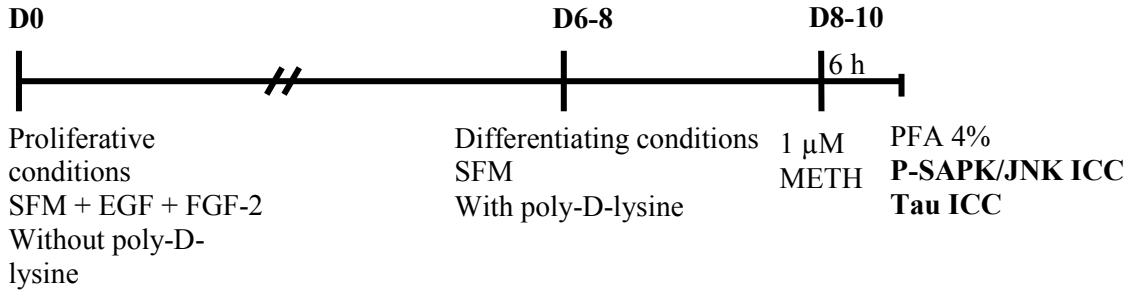
Figure 3.8. METH decreases neuronal differentiation in SVZ cell cultures. (A) Representative confocal photos of SVZ cell nuclei in SVZ cell cultures in the absence (control) or presence of 1 µM or 10 µM METH and labelled for NeuN (red). **(B)** Bar graph depicts the numbers of NeuN-positive cells, expressed as percentages of the total number of nuclei per culture, in treated and non-treated cultures. Data are expressed as a mean ± SEM ($n = 2$ independent experiments). * $p < 0.05$ using one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test for comparison with SVZ control cultures. NeuN, neuronal nuclear protein.

We then determined whether METH alters neuronal maturation, precisely whether METH affects neurite outgrowth and especially axonogenesis. The neuronal maturation goes through different stages in which a critical transition in neuron development is the formation of the axon. Oliva and collaborators (2006) showed that the phosphorylation of the stress-activated kinase (SAPK), also called c-Jun-NH_2-terminal kinase (JNK) is required for axonogenesis. They demonstrated that the phosphorylated form of JNK (P-JNK) is enriched in the developing axon and that the inhibition of JNK selectively prevents axon formation. Given this, immunolabelling of P-JNK-positive ramifications constitutes a reliable method to study and detect axonogenesis and neurite outgrowth. Moreover, previous studies performed in our laboratory showed a robust P-SAPK/JNK immunoreactivity in growth-cone like projections and in neurites emerging from the neurospheres in SVZ cultures treated for 6 hours with pro-neurogenic factors (Agasse et al., 2008; Bernardino et al., 2008).

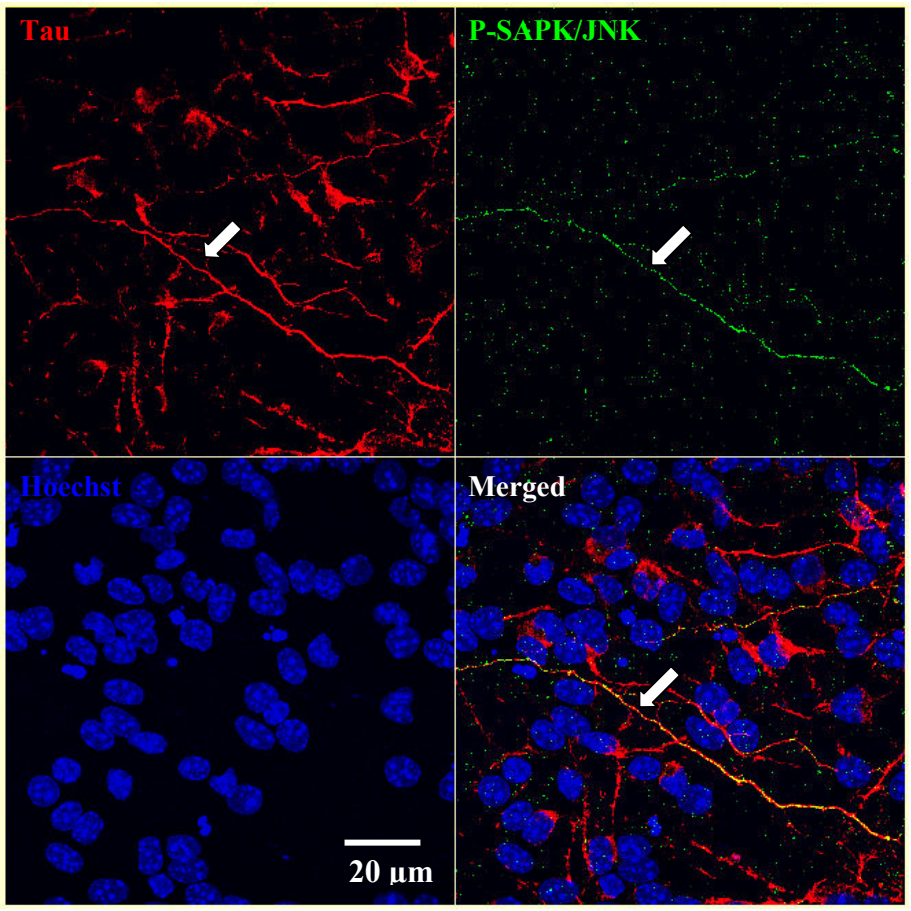
To investigate the effect of METH on axonogenesis, 6-8 day-old SVZ neurospheres were adhered for 48 hours onto poly-D-lysine coated coverslips and incubated with a non-toxic concentration of METH for 6 hours (Fig. 3.9A). Growing axons were labelled with the rabbit polyclonal anti-phosphorylated form of stress-activated protein kinase (anti-P-SAPK)/c-Jun-NH_2-terminal kinase (JNK) antibody. To ascertain that P-SAPK/JNK localization was associated with axons, double labelling immunocytochemistry was performed to visualize both P-SAPK/JNK and tau, a microtubule-associated protein that induces bundling and stabilization of axonal microtubules, found in developing and mature neurons (Jiménez-Mateos et al., 2006; Hong et al., 2008). A representative confocal photo of a non-treated SVZ cell culture illustrates a tau- and P-SAPK/JNK-positive developing axon (Fig. 3.9B). A diffuse P-SAPK/JNK staining throughout the cytoplasm was observed in the control cultures and also in the treated cultures. In the SVZ cell cultures treated with METH was observed a reduced branching of neuronal processes, and interestingly no clear distinction of an axon-like process could be observed since neurites were almost equal in length.

Axonogenesis was evaluated by quantification of the total length of tau- and P-SAPK/JNK-positive ramifications per neurosphere and also the total numbers of ramifications per neurosphere. For a non-toxic concentration METH decreased the total length of ramifications per neurosphere in SVZ cell cultures (Control: 228.70 ± 23.49 μm, 1 μM METH: 123.90 ± 20.20 μm; Fig. 3.9C). A non-significant decrease in the total numbers of ramifications per neurosphere in the treated SVZ cell cultures was observed (Control: 2.01 ± 0.11, 1 μM METH: 1.28 ± 0.30; Fig. 3.9D). Hence, METH impairs the formation of new axons.

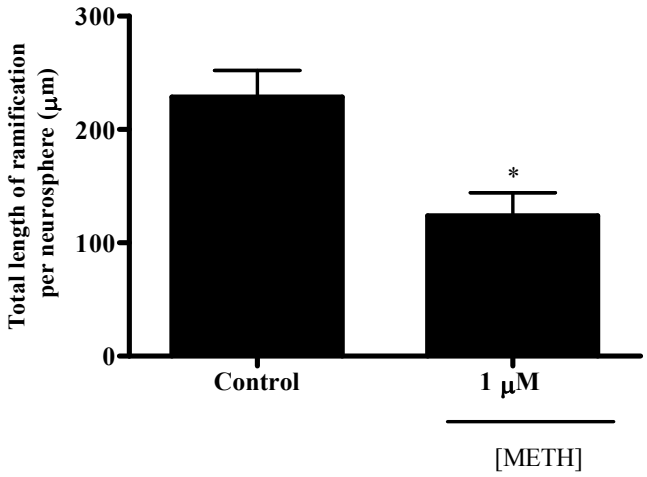
A)



B)


Tau
P-SAPK/JNK
Hoechst
Merged
20 µm

C)


Total length of ramification per neurosphere (µm)
300
200
100
0
*
Control
1 µM
[METH]

D)

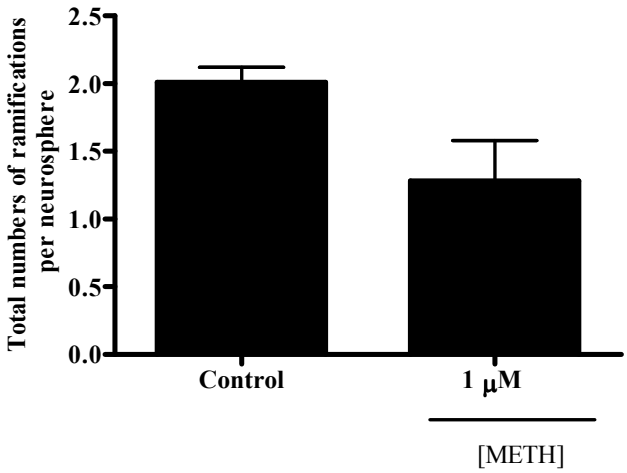


Figure 3.9. (A) Scheme for experimental protocol. **(B)** Representative confocal photo of non-treated SVZ cells labelled for P-SAPK/JNK (green) and Tau (red). **(C) METH decreases axonogenesis.** Bar graph depicts the total length of ramification (μm) per neurosphere in treated and non-treated cultures. **(D)** Bar graph depicts the total numbers of ramifications per neurosphere in treated and non-treated cultures. Data are expressed as a mean ± SEM (n = 2 independent experiments). * $p < 0.05$ using the unpaired student's t test for comparison with SVZ control cultures. P-SAPK/JNK, phosphorylated form of stress-activated protein kinase/ c-Jun-NH_2-terminal kinase.

CHAPTER 4

Discussion

The main objective of this work was to study *in vitro* the toxic effects of methamphetamine (METH) on subventricular zone (SVZ) neurogenesis in murine SVZ cell cultures and to unveil some of the underlying mechanisms.

As the majority of the reports upon METH point to a toxic effect of the drug, we first investigated cell survival in SVZ cultures challenged with METH. As described by Kelly and collaborators (2003), cell death by necrosis and apoptosis can be discriminated thank to the use of the propidium iodide uptake and the TUNEL staining. Using these specific assays, we found that METH induces cell death in SVZ cell cultures in a concentration-dependent manner both by necrosis and apoptosis. METH causes necrosis in the SVZ cell cultures at 500 μM, the highest concentration tested. However, in this situation, we observed that the levels of apoptosis remained close to control, which is probably due to the outcome of massive necrosis. In fact, at this high toxic concentration cells are killed rapidly and do not enter in apoptosis. Nevertheless, for the rest of the concentrations tested apoptosis prevails upon necrosis. Moreover, we demonstrated that METH specifically triggers apoptosis as the pro-apoptotic effect is prevented by the addition of the general specific caspase inhibitor ZVAD to the SVZ cell cultures treated with a toxic concentration of METH. In accordance, Zhu and collaborators showed an increase in the number of TUNEL-positive cells in the striatum of mice, as well as an increase in the death of striatal projection neurons, cholinergic and GABA-parvalbumin interneurons (Zhu et al., 2005 and 2006). Additionally, in an acute model of METH-induced neurotoxicity, where mice were injected with a single dose in the olfactory bulb, Deng and coworkers (2007) showed an increase in the TUNEL-positive neurons together with an increase in the levels of the pro-apoptotic proteins Bax and Bid and a decrease in the levels of the anti-apoptotic protein Bcl-2 in the olfactory bulb.

METH was proved to be toxic to different brain regions and cell populations. It is well known that this drug induces loss of pyramidal neurons and interneurons in the cortex and also damages the dopaminergic and serotoninergic neurons, as well as the projection neurons form the CA3 and CA1 regions of the hippocampus (for review, see Krasnova and Cadet, 2009). Moreover, in the present study, we also discriminated which cellular populations were affected by the drug. Indeed, SVZ cultures are heterogeneous and composed of neurons, astrocytes, oligodendrocytes and stem/progenitor cells in different maturation stages. We disclosed a putative selective toxicity of METH towards stem/progenitor cells (expressing SOX2) and neuroblasts (labelled for DCX). METH triggers stem/progenitor cell death, however, the number of DCX- and TUNEL-double-positive cells in METH treated and non-treated cultures are similar indicating that METH does not selectively kills immature neurons. To date, no studies report a preferential METH-induced toxic effect on SVZ stem/progenitor cells. A non significant increase in the TUNEL-positive immature neurons co-expressing DCX was found in this investigation. In accordance, Mandyam and co-workers (2008) showed no effect of METH intake on DCX-immunoreactive cells of rat SGZ. In addition, a study with the psychostimulant cocaine revealed that cocaine self-administration in rats do not decrease the numbers of immature neurons labelled with DCX in the SGZ (Noonan et al., 2008).

According to our and other data, it is clear that METH is toxic, but the mechanisms of METH-induced toxicity in SVZ cells are not yet known. Indeed, there are several questions raised by the present study that will be answered in a near future project. Even though, a lot of knowledge was already achieved concerning the mechanisms of METH-induced cell death in other models and rely mainly on dopamine, glutamate and calcium deregulation (for review see Krasnova and Cadet, 2009). As a lipophylic molecule, METH diffuses into the cells leading to a highly increase of intracellular calcium resulting in mitochondria disruption that can outcome in depletions in the levels of ATP and further necrosis (Li et al., 2008). Indeed, METH penetrates into intracellular organelles, like mitochondria, dissipating the electrochemical gradient which leads to cell apoptosis (Davidson et al., 2001). In specific, lost of the mitochondria membrane potential results in the release of mitochondrial proteins such as cytochrome c, apoptosis-inducing factor (AIF) and Smac/Diablo that initiate

apoptosis (Jayanthi et al., 2004; for review see Krasnova and Cadet, 2009). Of note, necrosis and apoptosis can occur in combination or sequentially, rarely the processes occur alone (Davidson et al., 2001). Additionally, METH decreases levels of anti-apoptotic proteins Bcl-2, Bcl-X_L while increasing pro-apoptotic Bax, Bad and Bid (Jayanthi et al., 2001; Deng et al., 2002).

In addition to mitochondrial dysfunction, METH toxicity is caused by the rapid and excessive release of dopamine (DA). METH enters dopaminergic neurons and provokes the release of DA from the synaptic vesicles into cytoplasm and by reverse transport into the synaptic cleft. Shortly after administration, METH dramatically increases DA levels (Brown et al., 2000). In the cytoplasm, DA rapidly auto-oxydizes to form toxic substances, such as superoxide radicals, hydroxyl radicals, hydrogen peroxide and DA quinones. Moreover, via activation of the DA receptors, METH may contribute to cell death. Indeed, antagonists for D1 (SCH23390) or D2 (sulpiride, eticlopride, raclopride) receptors reduce METH-induced cell death in the striatum (Jayanthi et al., 2005; Xu et al., 2005; Kranova and Cadet, 2009). Given that SVZ-derived cells *in vivo* can display a dopaminergic phenotype (Arrias-Carrión et al., 2006) the release of DA following METH treatment possibly mediates the observed cell death. In the future, we intend to clarify the possible involvement of DA in mediating the pro-apoptotic effects of METH.

Data in the literature also show that METH can trigger excitotoxicity mediated by the excessive release of glutamate. Additionally, glutamate receptor antagonists are proved to reduce METH-induced neurodegeneration of dopaminergic and serotoninergic neuronal terminals. Indeed, excessive release of this excitatory neurotransmitter might hyperactivate NMDA and AMPA receptors, leading to a deregulation of calcium homeostasis and formation of potentially toxic substances like superoxide radicals and nitric oxide that in combination form peroxynitrite, damaging the cells (for review see Cadet et al., 2007; Krasnova and Cadet, 2009). Since glutamate receptors play a key role in neurogenesis both in the SGZ and the SVZ (Brazel et al., 2005; Nacher et al., 2006), we will disclose the possible involvement of glutamate in mediating the pro-apoptotic effects of METH. Characterization of the expression of glutamate receptors in the SVZ cell cultures is already being performed in our laboratory.

Deregulation of the endoplasmic reticulum (ER) function, which is the intracellular store for Ca^{2+}, seems to be also involved in METH-induced toxicity. A high release of Ca^{2+} from the ER initiates calcium-dependent apoptosis. Indeed, METH increases calpain protease activity (a calcium responsive cytosolic protease involved in ER dependent apoptosis) that cleaves cytoskeletal molecules such as tau (Warren et al., 2007). In parallel, METH increases also the levels of caspase-12, glucose-regulated protein 78kD (GRP78) and CHOP that participate in ER dependent apoptosis (Jayanthi et al., 2004; Marciniak and Ron, 2006). We plan to clarify whether METH-induced cell death is due to calcium deregulation and will monitor calcium levels using the single cell calcium imaging technique in cells treated with METH as compared to non-treated cells.

Very recently, Tian and colleagues (2009) showed that besides the toxic effect of METH in rat hippocampal neural progenitor cell cultures, this drug of abuse also inhibits proliferation. However, the authors did not clarify whether this is due to a direct effect on cell proliferation rather than a consequence of the METH pro-apoptotic effect, as they measured proliferation using toxic concentrations of METH. In fact, we also observed that METH decreases BrdU incorporation at 48 hours in the SVZ for concentration of and above 100 μM. However, as METH increases cell death at these concentrations, the decrease of BrdU incorporation is probably due to the toxic effect rather than to a dynamic inhibition of proliferation. But to conclude on the effect of METH on cell proliferation, we carefully looked at the levels of BrdU incorporation for non-toxic concentrations of METH. Therefore, no differences in the numbers of BrdU-positive cells were observed at non-toxic concentrations as compared to control, which led to conclude that METH does not affect cell proliferation. Nevertheless, to disclose whether an inhibition of proliferation could concomitantly occur together with cell death at toxic concentrations, it is necessary to evaluate proliferation in cultures treated with METH and the inhibitor of apoptosis ZVAD. In this way, if a significantly decrease in the BrdU incorporation is observed when apoptosis is inhibited we can guarantee that METH inhibits proliferation. Indeed, several studies claim that METH inhibits proliferation of neural cells in the hippocampus of gerbils and mice (Hildebrandt et al., 1999; Teuchert-Noodt et al., 2000; Maeda et al., 2007). However, no detection of cell death is performed in these studies and, consequently, it is difficult to

conclude that METH is actually having an inhibitory effect on cell proliferation. It is noteworthy that in a paradigm of short access to the drug, a decrease in proliferation together with an increase in cell death was observed in the rat DG (Mandyam et al., 2008). Moreover, the psychostimulant cocaine at a non-toxic concentration is able to decrease proliferation of fetal human cortical cells, an effect mediated by a down-regulation of the expression of the cyclin A, a protein required for the G1 to S phase transition (Lee et al., 2008). To finally disclose whether METH decrease proliferation, levels of cyclin D1, a protein required for cell cycle G1/S transition which is expressed by SVZ cells *in vitro* (Coronas et al., 2004), will be evaluated in control and METH treated cultures.

In this work, we also showed that METH impedes neuronal differentiation. Neurons seem to be primarily affected by METH and this effect may be due to the inhibition of neuronal differentiation. Mandyam and collaborators (2008) reported that daily access to METH alters *in vivo* neurogenesis and neuronal maturation in the SGZ of the dentate gyrus in the hippocampus. Also, another study performed by Maeda and colleagues (2007) reported that phencyclidine (PCP), a synthetic hallucinogenic and psychostimulant, decreases neurogenesis and suggested a decrease in the total amount of mature neurons using an *in vivo* model with administration of non-toxic doses. Given all the accumulated data, we hypothesized that METH would be inhibiting differentiation in SVZ cell cultures. Indeed, the numbers of NeuN-positive neurons significantly decreased when a non-toxic concentration of METH was added to the SVZ cell cultures. Our studies revealed that METH affects neuronal maturation, precisely METH impedes axonogenesis as P-JNK labelling was decreased for a non-toxic concentration. The phosphorylation of SAPK/JNK proved to be necessary to the formation of new axons in hippocampal neurons (Oliva et al., 2006). Noteworthy, the JNK pathway is involved in cell migration and neuronal polarization (Bogoyevitch and Kobe, 2006; Mingorance-Le Meur, 2006). Extracellular stimuli, such as serum, epidermal growth factor and transforming growth factor-β involved in the maintenance of neural progenitors' cell cultures *in vitro*, activate several MAP kinases that phosphorylate and activate JNK. The activated JNK in turn phosphorylates DCX and MAPs, promoting microtubule dynamics, thus enhancing neuronal migration. This signalling module is present in migrating neurons and in the marginal zone of the

developing cerebral cortex. Furthermore, DCX phosphorylated by JNK affects neurite outgrowth and neuronal motility (Gdalyahu et al., 2004; Huang et al., 2004; Reiner et al., 2004; Bogoyevitch and Kobe, 2006). Indeed, besides inhibiting neuronal differentiation and impeding axonogenesis, METH may be also affecting SVZ cell motility and migration probably in the migrating neurons. None is known about the effect of METH on neurons migration. In the immature and adult brain, neural progenitor transplantation studies have shown that cells are able to migrate towards areas of brain damage (Shin et al., 2000; Riess et al., 2002; Jin et al., 2005) and studies of endogenous progenitor cells in the adult brain have shown new cells in the damaged areas following several types of injury (Magavi et al., 2000; Parent et al., 2002; Jin et al., 2003 and 2006). We intend to further disclose the effect of METH in cell migration. Immature neurons will be immunolabelled with anti-PSA-NCAM and further quantified. Nevertheless, studies on migration remain necessary and so the Boyden-Chamber assay constitutes a task to consider in this work.

Although it remains to be addressed, METH could act at the transcription levels and decrease the levels of transcription factors necessary for neuronal differentiation and commitment. Among the possible transcription factors are Dlx2, NeuroD and Mash1. Dlx2 is a transcription factor involved in the development of neurons and Dlx-positive cells are the first progenitors to make terminal divisions and differentiate as neurons (Doetsch et al., 2002). *Mash1* gene has been found to be related to neuronal commitment and differentiation and there is evidence for cross-regulation between *Mash1* and the *Dlx* genes (Parras et al., 2007; Roybon et al., 2009). NeuroD is a transcription factor involved in a variety of developmental functions including cell fate determination, differentiation and neuron survival (Morrow et al., 1999; Roybon et al., 2009). No studies are yet available regarding the regulation of the transcription of Dlx2, Mash1 or NeuroD by METH or any other related psychostimulant drug. However, this METH-inducing specific inhibition of neuronal differentiation may occur by inhibition of these transcription factors. To dissect the molecular mechanisms adjacent to the effect of METH on neuronal differentiation in the SVZ the expression levels of the transcription factors NeuroD, Dlx2 and/or Mash1 will be analysed whether by Northern blotting or qPCR in control and METH treated cultures.

CHAPTER 5

Conclusions

In conclusion, we demonstrated that: (1) METH induces cell death in a concentration-dependent manner in SVZ cell cultures, both by apoptosis and necrosis, (2) METH-induced toxicity have primarily effects on stem/progenitor cells but not on immature migrating neurons, (3) METH does not affect proliferation, and (4) at non-toxic concentrations, METH decreases neuronal differentiation and axonogenesis in SVZ cell cultures.

5.1. Methamphetamine, SVZ and challenge of brain repair capacity

The stem/progenitor cells residing in the SVZ of the adult brain constitute the biggest pool of neural stem cells during adult life. Hence, SVZ cells promote functional recovery in several models of neurodegeneration. The reconstruction of neuronal circuits by transplantation of stem/progenitor SVZ cells represents a promising strategy for brain repair and treatment of some of the neurological disorders affecting a local population of neurons such as in Parkinson's or Huntington's diseases, stroke, head trauma and demyelinating diseases. According to our study, a straight correlation between METH consumption and lost of brain regeneration is easy to speculate. Indeed, Tavazoie and co-workers (2008) showed *in vivo* that stem/progenitor cells contact the vasculature at sites that lack astrocyte endfeet and pericyte coverage, a modification of the blood-brain barrier that is necessary for the maintenance of neurogenesis in the intact and damaged brain. Additionally, it is considered that direct contact between vessels and stem cells probably increases the susceptibility of SVZ stem/progenitor cells to be affected by METH-induced toxicity and damage. Afterwards, METH users might display a drastic depletion in their stock of repairing cells impairing cell replacement in the case of brain damage. With the purpose of developing efficient therapies based on

SVZ cells as a reservoir of replacing cells it is important to assure the maintenance of the pool of stem cells in the SVZ. For that reason, to understand the effect of METH in the SVZ niche *in vivo* is a critical task. Given that, we aim to study SVZ cell dynamic in mice injected with METH. In order to show whether METH decrease the number of stem/progenitor cells in the SVZ *in vivo*, we will notably assess the self-renewal capacity of SVZ-derived single cells. SVZ cells dissociated from control and METH intoxicated mice will be challenged in their capacity to form primary and secondary multipotent neurospheres.

5.2. Strategies to protect the source of stem/progenitor cells in the human adult brain

We showed that METH is toxic for stem/progenitors cells which may hamper brain regeneration. We also unveiled that METH induces massively cell death in neural population which is in accordance to other studies that determined that METH-induced neurotoxicity overwhelm in a huge loss of brain cells. Overall, treatments to counterbalance the neurodegeration and damage induced by METH are needed. Thiriet and co-workers showed that neuropeptide Y (NPY) protects against METH-induced neuronal apoptosis in the mouse striatum and maintains neuronal integrity during cellular stress, being considered a neuroprotective agent (Thiriet et al., 2005). NPY rescues glutamate-induced excitotoxicity caused by METH exposure in hippocampal organotypic cultures (Silva et al., 2003) and also NPY protects retinal neural cells from 3,4-methylenedioxymethamphetamine (MDMA), a toxic psychostimulant (Álvaro et al., 2008). Additionally, NPY has been proved to be a potent pro-neurogenic substance, promoting neuronal differentiation and improving brain regeneration both in the dentate gyrus and in the SVZ (Howell et al., 2005; Agasse et al., 2008). In our laboratory, we observed that an acute high dose of METH significantly increases the levels of NPY mRNA levels in the mice hippocampus. These results allow us to hypothesize that up-regulation of NPY expression following METH intoxication may result from an attempt to promote neuroprotection or cell replacement through neurogenesis. As a neuroprotective agent NPY has to be administrated before the METH exposure, and as a pro-neurogenic factor it should be administrated after or before the METH exposure.

Indeed, to improve the preventing effect it would be optimal to administrate before and after drug exposure. The glial cell line-derived neurotrophic factor (GDNF) has been proved to rescue cells from the METH-induced neurotoxicity (Cass et al., 1996 and 2006). Additionally, GDNF infused into the ischemic striatum stimulates SVZ neurogenesis and the recruitment of surrogate cells (Kobayashi et al., 2006). Also, GDNF treatment should be seen as a preventive treatment and administrated before METH exposure.

Several investigations report that antioxidant molecules are efficient in preventing METH-induced oxidative stress *in vitro*. Moreover, antioxidant defence systems play a role for detoxification of METH-induced toxicity by the scavenging of free radicals within the cell, since a significant decline in this system may result in the disruption of mitochondrial function. Zhang and coworkers (2009) have proved that *N*-acetylcysteine amide (NACA), a novel antioxidant, protects against METH-induced oxidative stress and neurotoxicity in immortalized human brain endothelial cells. Zinc pre-treatment was proved to provide mitochondria protection and rescue METH-induced neurotoxicity in a dopaminergic human neuroblastoma cell line (Ajjimaporn et al., 2008). Other antioxidants have been shown to reduce the damage induced by METH suppressing the ROS production, like vitamin E (Wu et al., 2007) or selenium (Kim et al., 1999; Imam and Ali., 2000). Interestingly, melatonin, a hormone secreted by the pineal gland, that also acts a direct free radical scavenger, reduces induction of Bax, cleaved caspase-3 and cell death in METH treated human neuroblastoma cultures (Wisessmith et al., 2009). Moreover, melatonin levels have a key role in modulating neurogenesis in the hippocampus as melatonin increased the number of new neurons derived from adult hippocampal neural precursor cells *in vitro* by promoting cell survival (Ramírez-Rodríguez et al., 2009). Pre-treatment with several antioxidants like NACA or Zinc or even with melatonin may account to protect the SVZ cells against METH-induced toxicity. Additionally, to improve protection treatment and pre-treatment should be performed along with METH consumption.

References

Abdipranot A, Wu S, Stayte S, Vissel B (2008). The role of neurogenesis in neurodegenerative diseases and its implications for therapeutic development. CNS Neurol Disord Drug Targets 7:187-210.

Abrous DN, Adriani W, Montaron MF, Aurousseau C, Rougon G, Le Moal M (2002). Nicotine self-administration impairs hippocampal plasticity. J Neurosci 22:3656-62.

Agasse F, Bernardino L, Kristiansen H, Christiansen SH, Ferreira R, Silva B, Grade S, Woldbye D, Malva JO (2008). Neuropeptide Y Promotes Neurogenesis in Murine Subventricular Zone. Stem Cells 26:1636-45.

Ajjimaporn A, Shavali S, Ebadi M, Govitrapong P (2008). Zinc rescues dopaminergic SK-N-SH cell lines from methamphetamine-induced toxicity. Brain Res Bull 77(6):361-6.

Altman J (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 137(4):433-57.

Alvarez-Buylla A, Ling CY, Nottebohm F (1992). High vocal center growth and its relation to neurogenesis, neuronal replacement and song acquisition in juvenile canaries. J Neurobio 23(4):396-406.

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683-6.

Álvaro AR, Martins J, Costa AC, Fernandes E, Carvalho F, Ambrósio AF, Cavadas C (2008). Neuropeptide Y protects retinal neural cells against cell death induced by ecstasy. Neuroscience 152:97-105.

Annual Report of the Portuguese Institute for Drugs and Dependence, 2007. Relatório Anual 2007 – A Situação do País em Matéria de Drogas e Toxicodependências. Instituto da Droga e da Toxicodependência, I. P. – Departamento de Monitorização, Formação e Relações Internacionais – Núcleo de Estatística / Núcleo de Publicações e Documentação.

Arias-Carrión O, Hernández-López S, Ibañez-Sandoval O, Bargas J, Hernández-Cruz A, Drucker-Colín R (2006). Neuronal precursors within the adult rat subventricular zone differentiate into dopaminergic neurons after substantia nigra lesion and chromaffin cell transplant. J Neurosci Res 84(7):1425-37.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8(9):963-70.

Balsa C, et al. (2008). II Inquérito Nacional ao Consumo de Substâncias Psicoactivas na População Portuguesa. 2007, Lisboa: CEOS/FCSH/UNL.

Bédard A, Parent A (2004). Evidence for newly neurons in the human olfactory bulb. Brain Res Dev Brain Res 151:159-68.

Bernardino L, Agasse F, Silva B, Ferreira R, Grade S, Malva JO (2008). Tumor necrosis factor-alpha modulates survival, proliferation, and neuronal differentiation in neonatal subventricular zone cell cultures. Stem Cells 26(9):2361-71.

Bogoyevitch MA, Kobe B (2006). Uses for JNK: the Many and Varied Substrates of the c-Jun N-Terminal Kinases. Microbiol Mol Biol Rev 70(4):1061-95.

Brady KT, Sinha R (2005). Co-occurring mental and substance use disorders: the neurobiological effects of chronic stress. Am J Psychiatry 162:1483-93.

Brazel CY, Nuñez JL, Yang Z, Levison SW (2005). Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. Neuroscience 131:55-65.

Brown JM, Hanson GR, Fleckenstein AE (2000). Methamphetamine rapidly decreases vesicular dopamine uptake. J Neurochem 74(5):2221-3.

Cadet JL, Krasnova IN, Jayanthi S, Lyles J (2007). Neurotoxicity of substituted amphetamines: molecular and cellular mechanisms. Neurotox Res 11:183-202.

Cass WA (1996). GDNF selectively protects dopamine neurons over serotonin neurons against the neurotoxic effects of methamphetamine. J Neurosci 16:8132- 39.

Cass WA, Peters LE, Harned ME, Seroogy KB (2006). Protection by GDNF and other trophic factors against the dopamine-depleting effects of neurotoxic doses of methamphetamine. Ann N Y Acad Sci 1074:272-81.

Chawla S, le Pichon T, et al. (2007). United Nations 2007 World Drug Report. Office on Drugs and Crime. Research and Analysis Section of UNODC.

Coronas V, Bantubungi K, Fombonne J, Krantic S, Schiffmann SN, Roger M (2004). Dopamine D3 receptor stimulation promotes the proliferation of cells derived from the post-natal subventricular zone. J Neurochem 91(6):1292-301.

Curtis MA, Penney EB, Pearson AG, van Roon-Mom WM, Butterworth NJ, Dragunow M, Connor B, Faull RL (2003). Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. Proc Natl Acad Sci USA 100:9023-7.

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C (2007). Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315:1243-9.

Davidson C, Gow AJ, Lee TH, Ellinwood EH (2001). Methamphetamine neurotoxicity: necrotic and apoptotic mechanisms and relevance to human abuse and treatment. Brain Res Rev 36:1-22.

Deng X, Cadet JL (2000). Methamphetamine-induced apoptosis is attenuated in the striata of copper–zinc superoxide dismutase transgenic mice. Brain Res 83:121-4.

Deng X, Cai NS, McCoy MT, Chen W, Trush MA, Cadet JL (2002). Methamphetamine induces apoptosis in an immortalized rat striatal cell line by activating the mitochondrial cell death pathway. Neuropharmacology 42(6):837-45.

Deng X, Ladenheim B, Jayanthi S, Cadet JL (2007). Methamphetamine administration causes death of dopaminergic neurons in the mouse olfactory bulb. Biol Psychiatry 61:1235-43.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo J, Alvarez-Buylla A (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703-16.

Doetsch F, Petreanu L, Caille I, Garcia-Verdugo J, Alvarez-Buylla A (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron 36:1021-34.

Doetsch F (2003). The glial identity of neural stem cells. Nature Neurosci 6(11):1127-1134.

Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ (2000). Opiates inhibit neurogenesis in the adult rat hippocampus. Proc Natl Acad Sci USA 97:7579-84.

Faiz M, Acarin L, Villapol S, Schulz S, Castellano B, Gonzalez B (2008). Substantial migration of SVZ cells to the cortex results in the generation of new neurons in the excitotoxically damaged immature rat brain. Mol Cell Neurosci 38:170-82.

Fleckenstein AE, Voltz TJ, Riddle EL, Gibb JW, Hanson GR (2007). New insights into the mechanism of action of amphetamines. Annu Rev Pharmacol Toxicol 47:681-98.

Fuchs E, Tumbar T, Guasch G (2004). Socializing with the neighbors: stem cells and their niche. Cell 116:769-78.

Gdalyahu A, Ghosh I, Levy T, Sapir T, Sapoznik S, Fishler Y, Azoulai D, Reiner O (2004). DCX, a new mediator of the JNK pathway. The EMBO Journal 23:823-32.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257-71.

Gotz M, Huttner WB (2005). The cell biology of neurogenesis. Nature Rev Mol Cell Biol 6:777-88.

Gould E, Reeves AJ, Graziano MS, Gross GC (1999). Neurogenesis in the neocortex of adult primates. Science 286: 548-52.

Hall PA, Watt FM (1989). Stem cells: the generation and maintenance of cellular diversity. Development 106:619-33.

Hamamoto DT, Rhodus NL (2008). Methamphetamine abuse and dentistry. Oral Dis 15(1):27-37.

Hendrickson H, Laurenzana E, Owens SM (2006). Quantitative determination of total methamphetamine and active metabolites in rat tissue by liquid chromatography with tandem mass spectrometric detection. AAPS J 8: E709-17.

Hildebrandt K, Teuchert-Noodt G, Dawirs RR (1999). A single neonatal dose of methamphetamine suppresses dentate granule cell proliferation in adult gerbils which is restored to control values by acute doses of haloperidol. J Neural Transm 106(5-6):549-58.

Hong X, Tian Q, Liu Y, Wei W, Wang JZ (2008). P3-386: Localization of phosphorylated tau protein in immature neurons of adult hippocampus. Alzheimer's and Dementia 4(4,1):T634.

Howell OW, Doyle K, Goodman JH, Scharfman HE, Herzog H, Pringle A, Beck-Sickinger AG, Gray WP (2005). Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. J Neurochem 93(3):560-70.

Huang C, Jacobson K, Schaller MD (2004). MAP kinases and cell migration. J Cell Sci 117:4619-28.

Imam SZ, Ali SF (2000). Selenium, an antioxidant, attenuates methamphetamine-induced dopaminergic toxicity and peroxynitrite generation. Brain Res 855(1):186-91.

Iversen L (2006). Neurotransmitter transporters and their impact on the development of psychopharmacology. Br J Pharmacol 147:S82-88.

Jayanthi S, Deng X, Bordelon M, McCoy MT, Cadet JL (2001). Methamphetamine causes differential regulation of pro-death and anti-death Bcl-2 genes in the mouse neocortex. FASEB J 15:1745-52.

Jayanthi S, Deng X, Noailles PA, Ladenheim B, Cadet JL (2004). Methamphetamine induces neuronal apoptosis via cross-talks between endoplasmic reticulum and mitochondria-dependent death cascades. FASEB J 8(2):238-51.

Jayanthi S, Deng X, Ladenheim B, McCoy MT, Cluster A, Cai NS, Cadet JL (2005). Calcineurin/NFAT-induced up-regulation of the Fas ligand/Fas death pathway is involved in methamphetamine-induced neuronal apoptosis. Proc Natl Acad Sci USA 102:868-73.

Jiang W, Zhang Y, Xiao L, Van Cleemput J, Ji SP, Bai G, Zhang X (2005). Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. J Clin Invest 115(11):3104-16.

Jiménez-Mateos E, González-Billaut C, Dawson HN, Vitek MP, Avila J (2006). Role of MAP1B in axonal retrograde transport of mitochondria. Biochem J 397:53-9.

Jin K, Sun Y, Xie L, Peel A, Mao XO, Batteur S, Greenberg DA (2003). Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Mol Cell Neurosci 24:171-89.

Jin K, Peel AL, Mao XO, Xie L, Cottrell BA, Henshall DC, Greenberg DA (2004). Increased hippocampal neurogenesis in Alzheimer's disease. Proc Natl Acad Sci USA 101: 343.

Jin K, Sun Y, Xie L, Mao XO, Childs J, Peel A, Logvinova A, Banwait S, Greenberg DA (2005). Comparison of ischemia directed migration of neural precursor cells after intrastriatal, intraventricular, or intravenous transplantation in the rat. Neurobiol Dis 18:366-74.

Jin K, Wang X., Xie L, Mao XO, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg DA (2006). Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci USA 103:13198-202.

Kaneko N. & K. Sawamoto (2009). Adult neurogenesis and its alteration under pathological conditions. Neuroscience Research 63:155-64.

Kelly KJ, Sandoval RM, Dunn KW, Molitoris BA, Dagher PC (2003). A novel method to detemine specificity and sensitivity of the TUNEL reaction in the quantitation of apoptosis. Am J Physiol Cell Physiol 284:C1304-18.

Kim HC, Jhoo WK, Choi DY, Im DH, Shin EJ, Suh JH, Floyd RA, Bing G (1999). Protection of methamphetamine nigrostriatal toxicity by dietary selenium. Brain Res 851(1-2):76-86.

Kobayashi T, Ahlenius H, Thored P, Kobayashi R, Kokaia Z, Lindvall O (2006). Intracerebral infusion of glial cell line-derived neurotrophic factor promotes striatal neurogenesis after stroke in adult rats. Stroke 37(9):2361-67.

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

Lee C, Chen J, Tsai S, Sanchez JF, Errico SL, Ammable R, Su T, Huestis MA, Shen J, Becker KG, Geller HM, Freed WJ (2008). A Mechanism for the inhibition of neural progenitor cell proliferation by cocaine. PLOS Med 5(6):987-1004.

Li X, Wang H, Qiu P, Luo H (2008). Proteomic profiling of proteins associated with methamphetamine-induced neurotoxicity in different regions of rat brain. Neurochem Int 52:256-64.

Lledo P, Allonso M, Grubb MS (2006). Adult neurogenesis and functional plasticity in neuronal circuits. Nature Rev Neurosci 7:179-93.

Lois C, Alvarez-Buylla A (1994). Long-distance neuronal migration in the adult mammalian brain. Science 264:1145-8.

Maeda K, Sugino H, Hirose T, Kitagawa H, Nagai T, Mizoguchi H, Takuma K, Yamada K (2007). Clozapine Prevents a Decrease in Neurogenesis in Mice Repeatedly Treated With Phencyclidine. J Pharmacol Sci 103:299-308.

Magavi SS, Leavitt BR, Macklis JD (2000). Induction of neurogenesis in the neocortex of adult mice. Nature 405, 951-5.

Mandyam CD, Wee S, Crawford EF, Eisch AJ, Richardson HN, Koob GJ (2008). Varied access to intravenous methamphetamine self-administration differentially alters adult hippocampal neurogenesis. Biol Psychiatry 64:958-65.

Marciaki SJ, Ron D (2006). Endoplasmic reticulum stress signaling in disease. Physiol Rev 86:1133-49.

McKetin R, Mattick RP (1998). Attention and memory in illicit amphetamine users: Comparison with non-drug-using controls. Drug Alcohol Depend 50:181-4.

Ming GL, Song H (2005). Adult neurogenesis in the mammalian central nervous system. Ann Rev Neurosci 28:223-50.

Mingorance-Le Meur A (2006). JNK Gives Axons a Second Chance. The Journal of Neuroscience 26(47):12104-5.

Morrow EM, Furukawa T, Lee JE, Cepko CL (1999). NeuroD regulates multiple functions in the developing neural retina in rodent. Development 126(1):23-36.

Nacher J, McEwen BS (2006). The role of N-methyl-D-asparate receptors in neurogenesis. Hippocampus. 16:267-70.

Nait-Oumesmar B, Picard-Riera N, Kerninon C, Decker L, Seilhean D, Höglinger GU, Hirsch EC, Reynolds R, Baron-Van Evercooren A (2007). Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors. Proc Natl Acad Sci USA 104(11):4694-9.

Noonan MA, Choi KH, Self DW, Eisch AJ (2008) Withdrawal from Cocaine Self-Administration Normalizes Deficits in Proliferation and Enhances Maturity of Adult-Generated Hippocampal Neurons. J Neurosci 28(10):2516-26.

Oliva Jr AA, Atkins CM, Copenagle L, Banker GA (2006) Activated c-Jun N-Terminal Kinase Is Required for Axon Formation. J Neurosci 26(37):9462-70.

Ortega-Perez I, Murray K, Lledo P (2007). The how and why of adult neurogenesis. J Mol Hist 38:555-62.

Paulus MP, Hozack N, Frank L, Brown GG, Schuckit MA (2003). Decision making by methamphetamine-dependent subjects is associated with error-rate-independent decrease in prefrontal and parietal activation. Biol Psychiatry 53:65-74.

Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann Neurol 52:802-13.

Parras CM, Galli R, Britz O, Soares S, Galichet C, Battiste J, Johnson JE, Nakafuku M, Vescovi A, Guillemot F (2007). Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J 23:4495-4505.

Pencea V, Bingaman KD, Freedman LJ, and Luskin MB (2001). Neurogenesis in the subventricular zone and rostral migratory stream of the neonatal and adult primate forebrain. Exp Neurol 172:1-16

Pop C, Salvensen GS (2009). Human Caspases: Activation, Specificity and Regulation. J Biol Chem.

Privat A, Leblond CP (1972). The subependymal layer and neighboring region in the brain of the young rat. J Comp Neurol 146:277-302.

Quinton MS, Yamamoto BK (2006) Causes and consequences of Methamphetmine and MDMA toxicity. AAPS J 8(2):E337-47.

Rakic P (1985) Limits of neurogenesis in primates. Science 227: 1054-56.

Ramírez-Rodríguez G, Klempin F, Babu H, Benítez-King G, Kempermann G (2009). Melatonin modulates cell survival of new neurons in the hippocampus of adult mice. Neuropsychopharmacology.

Reiner O, Gdalyahu A, Ghosh I, Levy T, Sapoznik S, Nir R, Sapi T (2004) DCXs Phosphorylation by Not Just aNother Kinase (JNK). Journal of Cell Science 117:4619-28.

Riess P, Zhang C, Saatman KE, Laurer HL, Longhi LG, Raghupathi R, Lenzlinger PM, Lifshitz J, Boockvar J, Neugebauer E, Snyder EY, McIntosh TK (2002). Transplanted neural

stem cells survive, differentiate, and improve neurological motor function after experimental traumatic brain injury. Neurosurgery 51:1043-52. Discussion 1052-44.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-10.

Riquelme PA, Drapeau E, Doetsch F (2008). Brain micro-ecologies: neural stem cell niches in the adult mammalian brain. Phil Trans R Soc B 363:123-37.

Roybon L, Deierborg T, Brundin P, Li JY (2009). Involvement of Ngn2, Tbr and NeuroD proteins during postnatal olfactory bulb neurogenesis. Eur J Neurosci 29(2):232-43.

Salo R, Nordahl TE, Natsuaki Y, Leamon MH, Galloway GP, Waters C (2007). Attentional control and brain metabolite levels in methamphetamine abusers. Biol Psychiatry 61:1272-80.

Salo R, Ursu S, Buonocore MH, Leamon MH, Carter C (2009). Impaired prefrontal cortical function and disrupted adaptive cognitive control in methamphetamine abusers: a functional magnetic resonance imaging study. Biol Psychiatry 65:706-9.

Sanai N, Tramontin AD, Quinones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, Lawton MT, McDermott MW, Parsa AT, Manuel-Garcia Verdugo J (2004). Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427:740-44.

Sanai N, Berger M, Manuel-Garcia Verdugo J, Alvarez-Buylla A (2007). Comment on "Human Neuroblasts Migrate to the Olfactory Bulb via a Lateral Ventricular Extension". Science 318(5849):393.

Sayre LM, Perry G, Smith MA (2008). Oxidative stress and neurotoxicity. Chem Res Toxicol 21(1):172-88.

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, Goldm MS, Cadet JL (2008). Methamphetamine causes microglial activation in the brains of human abusers. J Neurosci 28(22):5756-61.

Shen Q, Wang Y, Kokovay E, Lin G, Chuang S, Goderie S, Roysam B, Temple S (2008). Adult SVZ Stem Cells Lie in a Vascular Niche: A Quantitative Analysis of Niche Cell-Cell Interactions. Cell Stem Cell 3:289-300.

Shin JJ, Fricker-Gates RA, Perez FA, Leavitt BR, Zurakowski D, Macklis JD (2000). Transplanted neuroblasts differentiate appropriately into projection neurons with correct neurotransmitter and receptor phenotype in neocortex undergoing targeted projection neuron degeneration. J Neurosci 20:7404-16.

Silva AP, Pinheiro PS, Carvalho AP, Carvalho CM, Jakobsen B, Zimmer J, Malva JO (2003). Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures. FASEB J 17:1118-20.

Sulzer D, Sonders MS, Poulsen NW, Galli A (2005). Mechanisms of neurotransmitter release by amphetamines: a review. Prog Neurobiol 75:406-33.

Tavazoie M, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo M, Doetsch F (2008). A specialized vascular niche for adult neural stem cells. Cell Stem Cell 3:279-88.

Teuchert-Noodt G, Dawirs RR, Hildebrandt K (2000). Adult treatment with methamphetamine transiently decreases dentate granule cell proliferation in the gerbil hippocampus. J Neural Transm 107(2):133-43.

Thiriet N, Deng X, Solinas M, Ladenheim B, Curtis W, Goldberg SR, Palmiter RD, Cadet JD (2005). Neuropeptide Y protects against mehamphetamine-induced neuronal apoptosis in the mouse striatum. J Neurosci 25(22):5273-9.

Thompson PM, Hayashi KM, Simon SL, Geaga JA, Hong MS, Sui Y (2004). Structural abnormalities in the brains of human subjects who use methamphetamine. J Neurosci 24:6028-36.

Tian C, Murrin LC, Zheng JC (2009). Mitochondrial Fragmentation Is Involved in Methamphetamine-Induced Cell Death in Rat Hippocampal Neural Progenitor Cells. Plos One 4(5):E5546.

Warren MW, Zheng W, Kobeissy FH, Cheng Liu M, Hayes RL, Gold MS, Larner SF, Wang KK (2007). Calpain- and caspase-mediated alphaII-spectrin and tau proteolysis in rat cerebrocortical neuronal cultures after ecstasy or methamphetamine exposure. Int J Neuropsychopharmacol 10:479-489.

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. Development 128:3759-3771.

Wisessmith W, Phansuwan-Pujito P, Govitrapong P, Chetsawang B (2009). Melatonin reduces induction of Bax, caspase and cell death in methamphetamine-treated human neuroblastoma SH-SY5Y cultured cells. J Pineal Res 46(4):433-440.

Wu CW, Ping YH, Yen JC, Chang CY, Wang SF, Yeh CL, Chi CW, Lee HC (2007). Enhanced oxidative stress and aberrant mitochondrial biogenesis in human neuroblastoma SH-SY5Y cells during methamphetamine induced apoptosis. Toxicol Appl Pharmacol 220(3):243-51.

Xu W, Zhu JP, Angulo JA (2005). Induction of striatal pre- and postsynaptic damage by methamphetamine requires the dopamine receptors. Synapse 58(2):110-21.

Yamaguchi M, Suzuki T, Seki T, Namba T, Juan R, Arai H (2004). Repetitive cocaine administration decreases neurogenesis in adult rat hippocampus. Ann N Y Acad Sci 1025:351-62.

Zhang RL, Zhang ZG, Chopp M (2008). Ischemic stroke and neurogenesis in the subventricular zone. Neuropharmacology 55(3):345-52.

Zhang X, Banerjee A, Banks WA, Ercal N (2009). *N*-Acetylcysteine amide protects against methamphetamine-induced oxidative stress and neurotoxicity in immortalized human brain endothelial cells. Brain Res 1275(12):87-95.

Zhao C, Deng W, Gage FH (2008). Mechanisms and Functional Implications of Adult Neurogenesis. Cell 132:645-60.

Zhu JP, Xu W, Angulo JA (2005). Disparity in the temporal appearance of methamphetamine-induced apoptosis and depletion of dopamine terminal markers in the striatum of mice. Brain Res 1049:171-81.

Zhu JP, Xu W, Angulo JA (2006). Methamphetamine-induced cell death: selective vulnerability in neuronal subpopulations of the striatum in mice. Neuroscience 140:607-22.