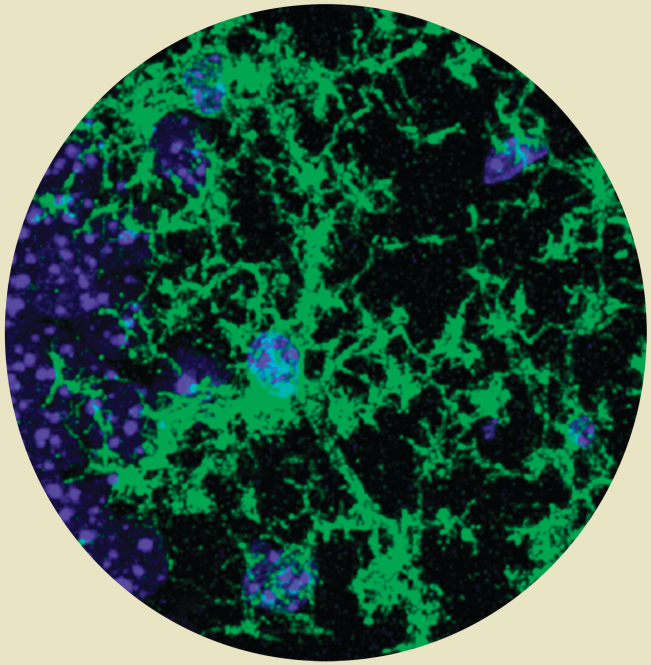
Joana Teresa Ferreira Gonçalves

METHAMPHETAMINE-INDUCED HIPPOCAMPAL DYSFUNCTION: THE ROLE OF PRO-INFLAMMATORY CYTOKINES AND NEUROPEPTIDE Y

Universidade de Coimbra



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Cover:

Mouse hippocampal microglial cells stained with macrophage-1 antigen (Mac-1 or CD11b). Fluorescence images were recorded on confocal microscope.

Joana Teresa Ferreira Gonçalves

O PAPEL DAS CITOCINAS PRÓ-INFLAMATÓRIAS E DO NEUROPEPTÍDEO Y NA DISFUNÇÃO DO HIPOCAMPO INDUZIDA PELA METANFETAMINA

METHAMPHETAMINE-INDUCED HIPPOCAMPAL DYSFUNCTION: THE ROLE OF PRO-INFLAMMATORY CYTOKINES AND NEUROPEPTIDE Y

2012

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para prestação de provas de doutoramento em Biociências.

Este trabalho foi realizado no Laboratório de Farmacologia e Terapêutica Experimental, Instituto Biomédico de Investigação da Luz e Imagem, Faculdade de Medicina, Universidade de Coimbra, Portugal, sob supervisão da Doutora Ana Paula Pereira da Silva Martins e da Professora Doutora Emília da Conceição Pedrosa Duarte, e no Rigshospitalet University Hospital, Departamento de Neurociências e Farmacologia, Universidade de Copenhaga, Dinamarca, sob supervisão do Doutor David P. Woldbye. A realização deste trabalho foi financiada ao abrigo da bolsa de Doutoramento SFRH/BD/35893/2007 da Fundação para a Ciência e a Tecnologia, co-financiada pelo POPH-QREN.

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INDEX

ABBREVIATIONS

AC	Adenylate cyclase
ACAMPs	Apoptotic cell associated molecular patterns
ADAMs	A densintegrins and metalloproteinases
ADHD	Attention deficit hyperactivity disorder
AIF	Apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
ATS	Amphetamine-type stimulants
Bad	Bcl-2-associated death promoter protein
Bak	Bcl-2 homologous antagonist/killer protein
Bax	Bcl-2-associated X protein
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
Bcl-X_L	B-cell lymphoma-extra large protein
Bcl-w	Bcl-2-like protein 2
Bcl-2	B-cell lymphoma 2 protein
BMVEC	Bovine microvessel endothelial cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CA1	*Cornu ammonis 1*
CA2	*Cornu ammonis 2*
CA3	*Cornu ammonis 3*
Ca^{2+}	Calcium
cDNA	Complementary DNA
cIAP-1	Cellular inhibitor of apoptosis protein 1
cIAP-2	Cellular inhibitor of apoptosis protein 2
CNS	Central nervous system
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CPON	C-terminal flanking peptide of NPY
DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine β-hydroxylase
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPP-4	Dipeptidyl peptidase-4
DTT	Dithiothreitol
D1R	Dopamine D1 receptor
D2R	Dopamine D2 receptor
D3R	Dopamine D3 receptor
ECF	enzymatic chemifluorescence reagent

ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular-signal-regulated kinase 1/2
FADD	Fas-associated protein with death domain
FBS	Fetal bovine serum
FDA	United State Food and Drug Administration
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBSS	Gey's buffered salt solution
GFAP	Glial fibrillary acidic protein
GIRK	G protein-coupled inwardly-rectifying potassium channel
GluR2	AMPA receptor GluA2
GPCRs	G protein-coupled receptors
gp130	Glycoprotein 130
Grb2	Growth-factor-receptor-bound protein
GTPγS	Guanosine 5'-O-[γ-thio]triphosphate
HAPI	Highly aggressively proliferating immortalized cells
HBSS	Hank's buffered salt solution
hCMEC/D3	Immortalized human brain microvascular endothelial cells
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICE	Interleukin-1 converting enzyme
i.c.v.	Intracerebroventricular
IDT	Instituto da Droga e Toxicodependência
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-1RAcP	IL-1R Accessory Protein
IRAK	IL-1R associated kinase
IL-1RI	Interleukin-1 type I receptor
IL-1RII	Interleukin-1 type II receptor
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
INDO	Indomethacin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
KO	Knockout
JAK/STAT	Janus Kinase/Signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
KA	Kainate
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharides
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mbIL-6R	Membrane-bound IL-6R
MCP-1	Monocyte chemotactic protein-1
MDMA	3,4-Methylenedioxymethamphetamine
METH	Methamphetamine
MHC-I	Major histocompatibility complex class I

MHC-II	Major histocompatibility complex class II
MIP-1α	Macrophage inflammatory protein-1α
MIP-1β	Macrophage inflammatory protein-1β
MMPs	Metalloproteinases
MMP-1	Metalloproteinase 1
MMP-2	Metalloproteinase 2
MMP-9	Metalloproteinase 9
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamicin
NADPH-d	Nicotinamide adenine dinucleotide phosphate-diaphorase
NE	Norepinephrine
NFκB	Nuclear factor-κB
NMDA	N-Methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOX	NADPH-oxidase
NPY	Neuropeptide Y
NR1	NMDA NR1 receptor
NR2A	NMDA NR2A receptor
NSAID	Non-steroidal anti-inflammatory drug
NTS	Nucleus tractus solitarius
ON	Overnight
PAMPs	Pathogen-associated molecular patterns
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PNS	Peripheral nervous system
PP	Pancreatic polypeptide
PRR	Pattern recognition receptor
PSD-95	Postsynaptic density-95
PVDF	Polyvinylidene fluoride
PYY	Peptide YY
p70S6K	70-kDa ribosomal protein S6 kinase
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.c.	Subcutaneous
SEM	Standard error of the mean
SERT	Serotonin transporter
SHP2	Src homology 2-containing tyrosine phosphatase

sIL-6R	Soluble IL-6R
SNAP-25	Synaptosome-associated protein of 25 000 daltons
solTNF	Soluble TNF
SOM	Somatostatin
SOS	Son of sevenless
SSC	Saline-sodium citrate
STAT3	Signal transducer and activator of transcription 3
sTNFR1	Soluble TNFR1
TACE	TNF-α converting enzyme
TH	Tyrosine hydroxylase
TIR	Toll/interleukin-1 receptor domain
TJs	Tight junctions
TLRs	Toll-like receptors
TMB	3,3',5,5'-tetramethylbenzidine
TMT	Trimethyltin
tmTNF	Transmembrane TNF
TNF-α	Tumor necrosis factor-α
TNFR1	TNF receptor type 1
TNFR2	TNF receptor type 2
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor-2
TRAF6	TNF receptor associated factor-6
Tuj-1	Beta III tubulin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UNODC	United Nations Office on Drugs and Crime
uPA	Urokinase-type plasminogen activator
VGlut1	Vesicular glutamate transporter 1
VMAT-2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
ZO-1	Zona occludens 1
Z-VAD	z-Val-Ala-Dl-Asp-(OMe)-fluoromethylketone
3-NT	3-Nitrotyrosine
5-HT	Serotonin
6-OHDA	6-hydroxydopamine

PUBLICATIONS

The results presented in this dissertation have been published or submitted for publication in international peer-reviewed journal:

Gonçalves J., Martins T., Ferreira R., Milhazes N., Borges F., Ribeiro C.F., Malva J.O., Macedo T.R., Silva A.P. (2008) Methamphetamine-induced early increase of IL-6 and TNF-alpha mRNA expression in the mouse brain. *Ann N Y Acad Sci.* 1139:103-11.

Gonçalves J., Baptista S., Martins T., Milhazes N., Borges F., Ribeiro C.F., Malva J.O., Silva A.P. (2010) Methamphetamine-induced neuroinflammation and neuronal dysfunction in the mice hippocampus: preventive effect of indomethacin. *Eur J Neurosci.* 31:315-26.

Coelho-Santos V.*, Gonçalves J.*, Ribeiro C.F., Silva A.P. (2012) Prevention of methamphetamine-induced microglial cell death by TNF-α and IL-6 through activation of the JAK/STAT pathway. *J Neuroinflammation* 9:103 (* These authors contributed equally to the work)

NOTE: Only the results relative to TNF system will be presented in this dissertation (Chapter 4).

Gonçalves J., Ribeiro C.F., Malva J.O., Silva A.P. (2012) Protective role of neuropeptide Y Y2 receptors in cell death and microglial activation following methamphetamine injury. *Eur J Neurosci.* (doi:10.1111/j.1460-9568.2012.08232.x).

Gonçalves J., Baptista S., Olesen M.V., Ribeiro C.F., Malva J.O., Woldbye D.W., Silva A.P. (2012) Methamphetamine-induced changes in the mice hippocampal neuropeptide Y system: implications for memory impairment. Under review in *J Neurochem.*

The present thesis also contributed to the following review:

Silva A.P., Martins T., Baptista S., Gonçalves J., Agasse F., Malva J.O. (2010) Brain injury associated with widely abused amphetamines: neuroinflammation, neurogenesis and blood-brain barrier. *Curr Drug Abuse Rev.* 3:239-54.

In this dissertation, the results presented from Chapter 2 to Chapter 6 are formatted as requested by the journal where the papers were published or submitted for publication, with minor modifications.

RESUMO

A metanfetamina (MET) é um psicoestimulante muito potente e altamente viciante que causa inúmeros problemas de saúde. De facto, os consumidores de MET apresentam disfunções cerebrais que podem desencadear doenças psiquiátricas e neurológicas. Na tentativa de melhor compreender as causas destes efeitos, vários estudos têm apontado os eventos intracelulares, tais como a oxidação da dopamina e a excitotoxicidade, como os principais responsáveis pela toxicidade desta droga. Recentemente, a inflamação foi também sugerida como outra possível causa da neurotoxicidade induzida pela MET, devido essencialmente ao aumento dos níveis de citocinas pró-inflamatórias. Contudo, não é claro o papel destas citocinas, visto que já foi demonstrado noutras condições patológicas que podem agravar o dano ou por outro lado ter um efeito protetor. Além disso, continua por esclarecer se a neuroinflamação é causa ou consequência das lesões cerebrais originadas pela MET.

O consumo de MET afeta negativamente não só o indivíduo mas também os seus familiares, amigos e sociedade em geral. No entanto, apesar do grande impacto sócio-económico que o consumo de MET constitui, existe muito pouca informação sobre terapias farmacológicas, sendo as terapias comportamentais o método mais eficaz até à data. Contudo, novos alvos terapêuticos têm sido identificados, tal como o neuropeptídeo Y (NPY), que foi descrito como neuroprotetor no hipocampo em condições de excitotoxicidade. Assim, alterações nos níveis de NPY e de citocinas pró-inflamatórias podem refletir uma tentativa de contrapor a degenerescência causada pela MET, desencadeando mecanismos de proteção e/ou regeneração.

Na tentativa de esclarecer algumas questões, começamos por investigar o efeito de uma dose única elevada de MET na produção de citocinas pró-inflamatórias, nomeadamente interleucina-1β (IL-1β), interleucina-6 (IL-6) e fator de necrose tumoral-α (TNF-α), no hipocampo, córtex frontal e estriado de

murganho. Verificámos que a MET induz uma resposta inflamatória rápida, com aumento dos níveis de RNA mensageiro (RNAm) da IL-6 nas três regiões cerebrais analisadas e do TNF-α apenas no hipocampo e estriado, sem qualquer alteração dos níveis da IL-1β.

Sendo o hipocampo particularmente suscetível à MET, fomos avaliar de que forma a microglia, astrócitos e neurónios do hipocampo de murganho respondem a esta droga. Verificámos que a administração de MET causa ativação da glia e, consequentemente, o aumento dos níveis proteicos do TNF-α e do seu recetor TNFR1, sem interferir na síntese de TNFR2. Neste modelo animal de intoxicação com MET, ocorre uma disfunção neuronal caracterizada pela diminuição da expressão da beta III tubulina (Tuj-1), sintaxina-1, calbindina D28K e tau e pelo aumento da proteína sinaptofisina, sem no entanto ocorrer morte celular. Também descobrimos que um anti-inflamatório não esteroide (indometacina) previne o processo neuroinflamatório e as alterações na expressão de Tuj-1 desencadeados pela administração de MET.

De forma a compreender qual o papel do TNF-α em condições de toxicidade induzida pela MET, expusemos uma linha celular de microglia e culturas organotípicas de fatias de hipocampo de murganho, a concentrações tóxicas desta droga. Observámos que a morte celular causada pela MET era acompanhada por uma rápida produção e libertação de TNF-α e por um aumento dos níveis proteicos do TNFR1. Concluímos também que o TNF-α endógeno não contribui para a toxicidade inerente à MET, e que por outro lado baixas concentrações exógenas desta citocina têm um efeito protetor quer no hipocampo quer nas células da microglia.

Posteriormente, explorámos qual o efeito da MET na função e níveis do NPY e seus recetores no hipocampo de murganho. Os resultados demonstraram que a MET provoca um aumento na expressão do NPY e dos recetores do subtipo Y_2 e Y_5. Verificámos também que a administração desta droga aumenta a ligação do NPY aos seus recetores na sub-região CA1, além de aumentar e diminuir a ativação seletiva do recetor Y_1 e Y_2, respetivamente. Mais ainda, apurámos que a MET provoca deterioração da memória de trabalho bem como

a de reconhecimento, envolvendo a disrupção da cascata de sinalização AKT/proteína alvo da rapamicina em mamíferos (mTOR). Porém, estes danos são prevenidos pelo bloqueio dos recetores Y_2.

Por outro lado, analisámos o papel neuroprotetor do NPY perante toxicidade induzida pela MET nos modelos *in vitro* referidos anteriormente. Demonstrámos que o NPY maioritariamente *via* recetor Y_2 tem um efeito protetor quer no hipocampo quer em células isoladas da microglia. Adicionalmente, observámos que a ativação do recetor Y_2 reduz a ativação da microglia causada pela MET nas culturas de fatias de hipocampo.

No seu conjunto, esta tese realça a importância do processo neuroinflamatório na disfunção cerebral induzida por MET, especialmente a nível do hipocampo. Por outro lado, este trabalho também fornece evidências que o NPY, e em particular o recetor Y_2, é um importante alvo celular para futuras abordagens terapêuticas.

Nota: Texto escrito conforme o Acordo Ortográfico - convertido pelo Lince

SUMMARY

Methamphetamine (METH) is a highly addictive psychostimulant, whose consumption is increasing worldwide causing serious health problems. Several studies have demonstrated that human METH abusers display brain dysfunctions that may contribute to psychiatric and neurological abnormalities. To date, most of the studies that explain the mechanisms underlying METH-induced neuropathology have focused on intra-neuronal events such as dopamine oxidation or excitotoxicity. However, recent evidence indicated that neuroinflammation and the consequent increase in pro-inflammatory cytokines could be implicated in METH neurotoxicity. It has been described in other pathologies that cytokines may have a dual role, but until now it is not clear how these inflammatory mediators can contribute to METH neuropathology. Besides, it remains to unravel whether the neuroinflammatory process is a cause or consequence of METH-induced brain injury.

The detrimental consequences of METH consumption are a public health problem that affects not only the individual but also the family, friends and society in general. However, despite the huge socio-economical impact of METH use, to date there is little information regarding pharmacological treatments, being behavioral therapies the most effective treatment. Interestingly, a new target has been raised as neuroprotector against METH-induced striatal cell death, the neuropeptide Y (NPY). In fact, pieces of evidence described that NPY acts as a neuroprotective agent in the hippocampus under excitotoxic insult. Thus, alterations in NPY and pro-inflammatory cytokines may reflect an attempt to counteract METH-induced degeneration by initiating protective and/or regenerative mechanisms.

In an attempt to clarify some issues, we started by investigating the effect of a single high dose of METH on the pro-inflammatory cytokines production, namely interleukine-1β (IL-1β), interleukine-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the mice hippocampus, frontal cortex and striatum. It was found that METH induces a prompt inflammatory process with increase of IL-6 mRNA

levels in the three brain regions analyzed and TNF-α mRNA up-regulation only in hippocampus and frontal cortex, with no differences in IL-1β mRNA levels.

Since the hippocampus appears to be particularly susceptible to METH, our goal was to explore how METH acute treatment affects microglia, astrocytes and neurons, and to evaluate possible alterations in the TNF system in this brain region. Here, we detected that METH caused glial activation and, consequently, an increase of TNF-α and TNFR1 protein levels, without affecting TNFR2 expression. Furthermore, METH treatment leads to hippocampal neuronal dysfunction characterized by decrease in beta III tubulin (Tuj-1), syntaxin-1, calbindin D28k and tau protein levels, and synaptophysin expression enhancement, without cell death. We also concluded that indomethacin, an anti-inflammatory drug, prevented METH-induced glia activation and both TNF system and Tuj-1 changes.

To understand if TNF signaling was beneficial or detrimental under conditions of METH toxicity, we exposed mouse microglial cells and hippocampal organotypic slice cultures to a toxic concentration of the drug. We observed that METH-induced cell death was followed by a quick production and release of TNF-α, and TNFR1 protein levels up-regulation. However, the endogenous TNF-α did not contribute to METH-induced toxicity, but exogenous low concentrations of this cytokine had a protective effect against neuronal and microglial cell death mediated by the drug.

Afterwards, we explored the impact of METH on hippocampal NPY system, namely in NPY and its receptor levels and function. In conclusion, METH intoxication increased NPY, Y_2 and Y_5 expression in the mice hippocampal formation. Besides, METH also increased the total NPY binding in CA1 sub-region, and led to an up- and down-regulation of selective Y_1 and Y_2 receptor activation. Moreover, we showed that METH induced spatial working and recognition memories impairment and disrupted AKT/mammalian target of rapamycin (mTOR) pathway, which was prevented by with the blockade of Y_2 receptors.

We then analyzed the neuroprotective role of NPY under conditions of

METH toxicity in organotypic hippocampal slice and microglial cultures. In both models, we found that NPY mainly *via* Y_2 receptor signaling had a protective effect against METH-induced cell death. Additionally, the Y_2 receptor activation reduced microglial activation induced by METH in hippocampal slice cultures.

Taken together, the present thesis shows the relevance of the neuroinflammatory process in METH-induced neuropathology, especially in the hippocampus. Moreover, our data also provide evidence that NPY system, and particularly the Y_2 receptor, is an important target to take into consideration in future therapeutic approaches.

Chapter 1

Introduction

1.1. Methamphetamine

1.1.1. Methamphetamine consumption

Drug abuse is a serious health problem worldwide and the consumption of methamphetamine (*N*-methyl-*O*-phenylisopropylamine; METH) is one of the major public concerns because its abuse has increased dramatically in the last years, which have resulted in extensive public health, legal, social and environment problems. In fact, METH is widely abused, mainly by young people, due to its ability to increase alertness, concentration, energy, self-confidence and libido (Rawson *et al.*, 2007). METH belongs to the group of amphetamine-type stimulants (ATS) that also include amphetamine (1-methyl-2-phenethylamine; AMPH) and 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy"; (Fleckenstein *et al.*, 2007; Silva *et al.*, 2010; Steinkellner *et al.*, 2011). These compounds are comprised of a phenyl ring connected to an amino group by a two-carbon side chain with a methyl group on carbon-1 of the side chain (Figure 1.1), showing a similar molecular structure to the monoaminergic neurotransmitter dopamine (DA; Fleckenstein *et al.*, 2007; Steinkellner *et al.*, 2011).

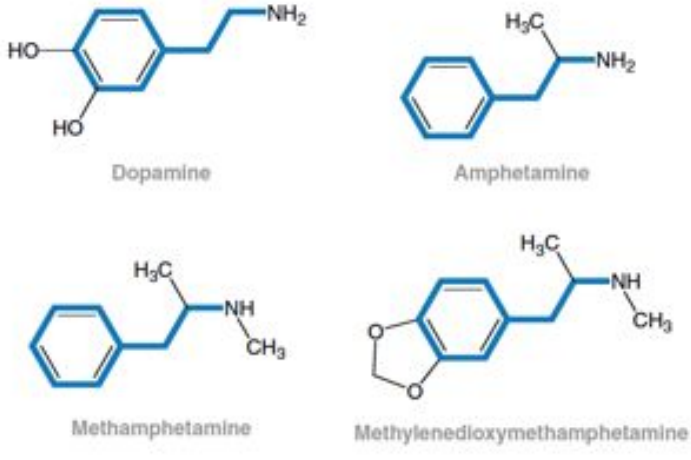


Figure 1.1. *Chemical structure of dopamine and amphetamine-type stimulants (ATS): methamphetamine (METH), amphetamine (AMPH) and 3,4-methylenedioxymethamphetamine (MDMA; Fleckenstein et al., 2007).*

METH can be found as a white, odorless, and bitter-tasting crystalline powder that is readily soluble in water or alcohol and can be injected or snorted. Pure crystals are usually used to smoke, and to ingest, the users buy it as big chunks, usually found in yellow, white, pink, brown and green (Figure 1.2). METH has several street names such as speed, crystal, ice or glass.

Figure 1.2. *METH can be presented to the users in several forms such as powder, crystal or rock.*

This drug of abuse was synthesized for the first time in 1893 in Japan by Nagayoshi Nagai and was widely used by Japanese, American and German military personal during World War II, to combat fatigue and increase performance (Meredith *et al.*, 2005). In 1944, U.S. Food and Drug Administration (FDA) approved METH prescription for the treatment of narcolepsy, mild depression, postencephalitic parkinsonism, chronic alcoholism, cerebral arteriosclerosis, attention-deficit hyperactivity disorder (ADHD) and the short-term management of exogenous obesity (Sulzer *et al.*, 2005). Nowadays, METH and METH-related medicines are only prescribed for obesity, narcolepsy and ADHD (Sulzer *et al.*, 2005) due to its highly addictive properties and the probability to originate several behavior dysfunctions like anxiety, depression, aggressiveness and even suicide together with anxiety and violent behavior (Berman *et al.*, 2008). Also, some recent studies have suggested that METH users may develop schizophrenia (McKetin *et al.*, 2010; Salo *et al.*, 2011).

The consumption of METH has increased over the last years, in part due to its simple synthesis, where most of necessary chemicals are over-the-counter products and usually available in household or allergy medicines. According to

the United Nations Office on Drugs and Crime (UNODOC) World Drug Report 2011 (United Nations, 2011), METH is ranked as the second most widely illegally manufactured, distributed and abused drug after cannabis. Moreover, this study estimates that in 2009 there were between 13.7 and 56.4 million people aged 15-64 globally who used amphetamines. In Europe, the annual prevalence of amphetamines consumption is estimated between 2.6 to 3.3 million people who had used these substances and, like in other regions of world, the majority of amphetamines users fall within the 15-64 years age group. Besides, the prevalence of these drugs is, overall, higher in West and Central Europe than in East and South-East Europe (United Nations, 2011). Furthermore, METH consumption is increasing in popularity among college students, women and young professionals (Gettig *et al.*, 2006). Also, some studies suggest that METH use is more common in gay and bisexual men than in the general population, which is associated with more frequent sexual risk behaviors and increased risks for human immunodeficiency virus (HIV) transmission (Shoptaw, 2006). In Portugal, according to the report from the "Instituto da Droga e da Toxicodependência, IDT 2009", between 2001 and 2007 there was an increase in amphetamines consumption from 0.5 % to 0.9 % in the total population and from 0.6 % to 1.3 % in young adult. Currently, there is no pharmacological therapy with established efficacy for the treatment of METH addiction, or any medication approved by the regulatory authorities for such pathology (Karila *et al.*, 2010).

1.1.2. Methamphetamine toxicity in the central nervous system

Chronic abuse of METH causes severe health complications, such as neurological and psychiatric abnormalities. Indeed, it is well documented that METH abusers show deficits in learning and working memory, and also in attention together with delayed recall and processing speed (Simon *et al.*, 2002, 2010; Thompson *et al.*, 2004; Woods *et al.*, 2005; Salo *et al.*, 2009). Similarly to Parkinson's disease (Kanthasamy *et al.*, 2010) and schizophrenia (Jentsch *et al.*, 2000), the abnormal dopaminergic (DAergic) system is a characteristic of METH-

induced neuropathology. In fact, several authors described that human METH abusers showed a persistent loss of dopamine transporters (DAT) density in *caudate-putamen*, *nucleus accumbens* and pre-frontal cortex that can be associated with the duration of METH use and its psychiatric symptoms (Sekine *et al.*, 2001, 2003; Chang *et al.*, 2007). Moreover, striatal reduction of DAT is accompanied by decrease of DA receptor D2 (D2) and vesicular monoamine transporter 2 (VMAT-2) (Chang *et al.*, 2007). Besides DAergic system, METH can also interfere with serotonergic pathways (Sora *et al.*, 2009) by decreasing the density of serotonin transporter (SERT) in midbrain, thalamus, cerebellum and orbitofrontal and occipital cortices (Sekine *et al.*, 2006; Chang *et al.*, 2007; Kish *et al.*, 2009).

Autopsy data from human METH abusers demonstrated that the deficits in DA and DAT levels are consistent with DAergic neuronal damage together with reduction in markers of neuronal integrity (Berman *et al.*, 2008). Moreover, positron emission tomography (PET) studies showed that glucose metabolism, an index of brain functional activity, was decreased in METH abusers (Chang *et al.*, 2007; Berman *et al.*, 2008). It is now clear that METH addiction leads to severe gray-matter deficits in the cingulate-limbic cortex and reduction of hippocampal volume, which seems to be correlated with cognitive impairment observed in METH users (Thompson *et al.*, 2004; Berman *et al.*, 2008; Figure 1.3). Moreover, this drug also originates significant white-matter hypertrophy and higher striatal volume that might reflect in compensatory responses to initial neurotoxicity resulting in altered myelination and adaptive glial changes (Thompson *et al.*, 2004; Berman *et al.*, 2008; Figure 1.3).

Other consequence of the ability of METH to increase DA-evoked long-term adaptive changes in DAergic transmission is the disturbance and desensitization of the reward system (Koob & Le Moal, 1997), which regulates and controls behavior by inducing pleasurable effects. This system consists of DAergic neurons of the ventral tegmental area (VTA) that project to the *nucleus accumbens*, amygdala and prefrontal cortex (Wise, 2009), and its activation by METH can produce addiction, which is a chronic and recurrent disease,

characterized by absolute dominance of drug-seeking behavior (Vetulani, 2001; Volkow *et al.*, 2011).

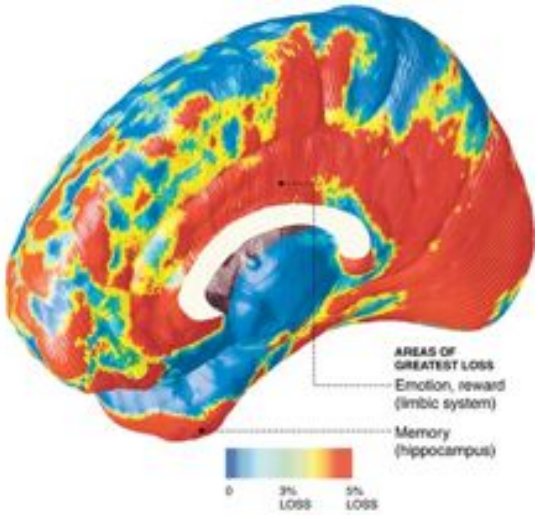


Figure 1.3. *Mapped brain changes caused by methamphetamine (METH) chronic consumption. The scale represents the average differences in brain tissue volume of METH users as compared with non-users (adapted from Thompson et al., 2004).*

Although several clinical studies validated that METH induces brain damage, the cellular mechanisms underlying its toxicity are far to be fully understood (Cadet & Krasnova, 2009; Silva *et al.*, 2010). Most of the studies have focused on oxidative stress, excitotoxicity and neuroinflammation (Cadet & Krasnova, 2009; Silva *et al.*, 2010).

1.1.2.1. Dopaminergic system dysfunction

METH has chemical structural similarity with DA (Figure 1.1) and it is a small lipophilic molecule, which allows the drug to enter DA neurons *via* DAT and passive diffusion, respectively (Cadet & Krasnova, 2009; Krasnova & Cadet, 2009). Under physiological conditions, neuronal activation leads to vesicular release of DA into the synaptic cleft, that will be then removed by DAT and stored in vesicles by VMAT-2, which protects DA from oxidation and degradation (Sulzer *et al.*, 2005; Volz *et al.*, 2007; Figure 1.4 and 1.5). The presence of METH in DA terminals deregulates the function of DAT and/or

VMAT-2 originating an abnormal release of DA (Krasnova & Cadet, 2009; Silva *et al.*, 2010; Figure 1.4).

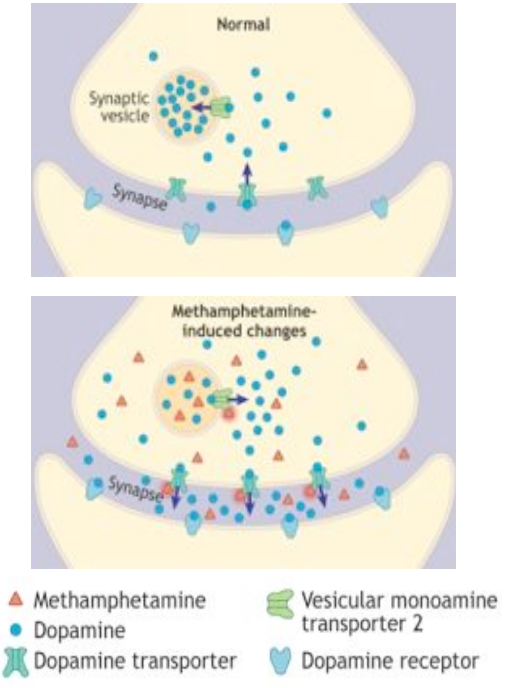


Figure 1.4. *Mechanisms underlying methamphetamine (METH)-induced dopamine overflow (Kish, 2008)*.

METH administration causes persistent DAergic deficits in rodents (Hadlock *et al.*, 2009; Krasnova & Cadet, 2009). Indeed, it was observed that neurotoxic regimen of METH [4x 4 mg/kg, 2h intervals; subcutaneously (s.c.)] induces a significant increase in rat striatal DA release, which was attenuated by DA receptor 1 (D1) and receptor 2 (D2) antagonists (O'Dell *et al.*, 1993). These observations clear show the involvement of DA receptors in METH neurotoxicity (Xu *et al.*, 2005). Concerning an acute treatment of METH (4 mg/kg; s.c.), it was described that a pretreatment (intrastriatal injection) of D1 or D2 receptor antagonist was able to prevent cortical and striatal METH toxicity as well as immediate early genes expression, namely Fos, in same brain regions (Gross & Marshall, 2009). Besides, Lee and collaborators (Lee *et al.*, 2009) evaluated METH-dependent subjects and found that, when compared

with healthy control subjects, they have reduced striatal D2/D3 receptor availability, which can be correlated with impulsive temperament. Other authors verified that mice treated with a single high dose of METH [30 mg/kg; intraperitoneally (i.p.)] show a depletion of DA terminal markers in striatum that begins to appear at 24h and reaches about 80% of decrease following 48h post-injection (Zhu *et al.*, 2005).

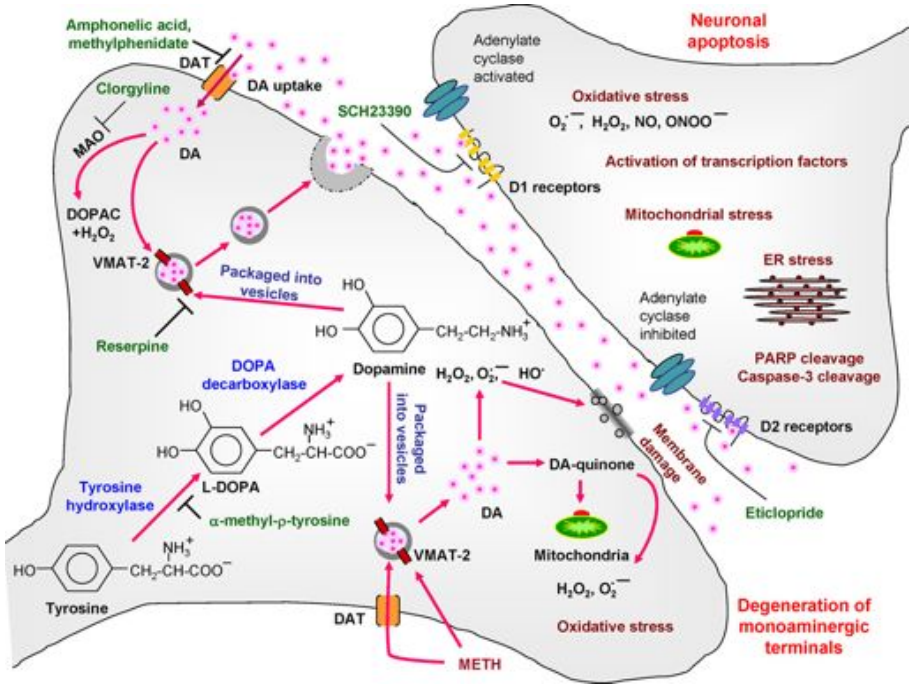


Figure 1.5. *Cellular and molecular events involved in methamphetamine (METH)-induced dopamine terminal degeneration and neuronal apoptosis within the striatum (Krasnova and Cadet 2009). Main abbreviations: DA, dopamine; DAT, dopamine transporters; DOPAC, 3,4-Dihydroxyphenylacetic acid; ER, endoplasmic reticulum; L-DOPA, L-3,4-dihydroxyphenylalanine; MAO, monoamine oxidase; VMAT-2, vesicular monoamine transporter 2.*

Furthermore, some authors reported that the striatal and cortical DAT density and tyrosine hydroxylase (TH) levels in rats are decreased following a extended (6-8h) access to METH self-administration, which is the better model to mimic human drug-taking behavior (Schwendt *et al.*, 2009; Krasnova *et al.*, 2010;

McFadden *et al.*, 2011). In Figure 1.5 are summarized the cellular and molecular events involved in METH-induced DAergic terminal degeneration.

1.1.2.2. Oxidative stress

One feature of METH neurotoxicity is its ability to prevent DA uptake into vesicles or to promote the efflux of vesicular DA into the cytoplasm, which results in the formation of DA quinones and superoxide radicals within nerve terminals (LaVoie & Hastings, 1999; Fleckenstein & Hanson, 2003; Figure 1.5). These events will lead to the production of reactive oxygen and nitrogen species (ROS and RNS, respectively), and lipid peroxidation (Fleckenstein *et al.*, 2007; Yamamoto *et al.*, 2010). In fact, Gluck and collaborators verified that a repeated METH treatment (4x 10 mg/kg, each to 2h; i.p.) resulted in formation of lipid and protein markers of oxidative stress in both mice hippocampus and striatum. Furthermore, other study reported that METH (5 or 15 mg/kg; i.p.) induced oxidative stress demonstrating a decrease of glutathione and increase of oxidized glutathione levels in rat cortex and striatum (Acikgoz *et al.*, 2001). Besides, *in vitro* studies have shown that acute METH exposure induces a temporal sequence of cellular events (Wu *et al.*, 2007). This work demonstrated that METH decreased mitochondrial membrane potential within 1h followed by extensive decline in mitochondrial membrane potential and ROS levels up-regulation after 8h of incubation, an increase in mitochondrial mass after 24h, and finally a decrease in mitochondrial DNA copy number and mitochondrial proteins as well as the occurrence of apoptosis after 48h of drug exposure (Wu *et al.*, 2007). In accordance with these findings there are several studies showing that METH neurotoxicity can be attenuated by free radical scavengers and antioxidants (Sharma *et al.*, 2007; Volz *et al.*, 2007). Moreover, changes in nitric oxide (NO) metabolism can also contribute to METH-induced oxidative stress and neurotoxicity (Itzhak & Ali, 2006). Indeed, both repeated (3x 5 mg/kg; i.p.) and acute (30 mg/kg; i.p.) METH administration leads to an up-regulation of nitrate concentration, neuronal nitric oxide synthase (nNOS) and 3-nitrotyrosine (3-NT) in mice striatum (Anderson & Itzhak, 2006; Wang &

Angulo, 2011). The involvement of NO in METH toxicity is also supported by findings demonstrating that in nNOS knockout (KO) mice there were no formations of 3-NT and DA terminal degeneration in striatum (Imam *et al.*, 2001). The presence of oxidative stress has also been documented in human METH users (Mirecki *et al.*, 2004; Fitzmaurice *et al.*, 2006).

1.1.2.3. Glutamatergic system deregulation

Glutamate (GLU) is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and its toxicity seems to be correlated with NO production because this molecule rapidly induces both depolarization and GLU release that, in turn, could lead to excitotoxic events (Brown, 2010). Thus, it was postulated that GLU can be involved in METH toxicity and this is supported by findings demonstrating that METH increases GLU release in rat striatum (Mark *et al.*, 2004; Mark *et al.*, 2007; Abekawa *et al.*, 2011), hippocampus (Rocher & Gardier, 2001) and VTA (Zhang *et al.*, 2001). It is well known that GLU is involved in several neurodegenerative diseases (Albrecht *et al.*, 2011; Bordji *et al.*, 2011; Kim *et al.*, 2011b) and METH neuropathology is not an exception. In fact, previously our group observed that an acute METH administration (30 mg/kg; s.c.) increased the rat striatal and cortical α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR2 protein levels together with a cortical N-methyl-D-aspartate (NMDA) receptor subunit NR1 and NR2A down- and up-regulation, respectively (Simões *et al.*, 2008). Concerning rat hippocampus, we verified that the same METH treatment increased GluR2 and NR2A protein levels without changes in NR1 subunit (Simões *et al.*, 2007). These results suggest that the alterations in AMPA and NMDA receptor levels can be a protective mechanism in order to counteract METH-induced toxicity, but consequently, may also explain, at least in part, memory impairment presented by these animals (Simões *et al.*, 2007, 2008). Accordingly, rats repeatedly treated with METH (4x 10 mg/kg, each 2h; s.c.) showed an up-regulation of function and expression of vesicular GLU transporter-1 (VGLUT1) in striatum and cortex (Mark *et al.*, 2007).

In vitro studies with hippocampal cultures also demonstrated that the inhibition of Ca^{2+} release from endoplasmic reticulum prevented METH-induced cell death, in part, due to a decrease of NMDA receptor activation and, subsequently, GLU release (Smith *et al.*, 2010). These findings were attested by studies demonstrating a protection from METH-induced striatal and cortical DAergic injuries by NMDA or AMPA receptor antagonists (Gross & Marshall, 2009; Gross *et al.*, 2011).

1.1.2.4. Hyperthermia and mitochondrial function impairment

Hyperthermia seems to be also a strong modulator of METH-induced long-term toxicity to 5-HT and DA terminals, because most of physicochemical processes that control neural activity are temperature-dependent (Kiyatkin, 2005; Yamamoto *et al.*, 2010). In fact, it was reported that the administration of multiple high doses of METH (4x 10 mg/kg; i.p.) at room temperature caused a significant depletion of DA in mice striatum, but when the same treatment was performed in a cold environment the striatal DA toxicity was blocked (Ali *et al.*, 1994). Since hyperthermia by itself does not lead to striatal DA depletion (Bowyer, 1995), it was suggested that this event might interact with other mediators of METH neurotoxicity, namely GLU neurotransmission up-regulation and oxidative stress (Yamamoto *et al.*, 2010). Indeed, pretreatment with NMDA receptor antagonists was able to reduce body temperature as well as protect against METH-induced depletion in 5-HT and DA content and tyrosine hydroxylase (TH) activity in mice striatum (Sonsalla *et al.*, 1991; Bowyer, 1995). Furthermore, blockade of METH-induced hyperthermia reduces the formation of ROS, and consequently mitigates the DA toxicity in rat striatum (Fleckenstein *et al.*, 1997). On the other hand, some recent studies have demonstrated that METH toxicity can be independent of body temperature (Thomas *et al.*, 2008; Herring *et al.*, 2010).

METH administration can also impair mitochondrial function (Krasnova & Cadet, 2009; Yamamoto *et al.*, 2010), as represented in Figure 1.6. More specifically, toxic doses of METH inhibit mitochondrial electron transport chain

enzyme complex I (Klongpanichapak *et al.*, 2006), complex II-III (Brown *et al.*, 2005) and complex IV (Burrows *et al.*, 2000) in rodent striatum and other DA-containing brain regions, which is associated with a reduction of adenosine-5'-triphosphate (ATP) stores in the brain (Burrows *et al.*, 2000). In turn, the decrease of ATP generation by mitochondrial deficits increases ROS production (Wu *et al.*, 2007). Moreover, mitochondria-dependent death pathway is involved in METH-related neuronal apoptosis (Cadet *et al.*, 2005; Krasnova & Cadet, 2009). Actually, a single high dose of METH (40 mg/kg; i.p.) caused apoptotic cell death in monoaminergic cells of mice frontal cortex through up-regulation of pro-apoptotic proteins, such as Bax, Bad, Bak and Bid, and reduction of anti-apoptotic molecules, namely Bcl-2, Bcl-X_L and Bclw (Jayanthi *et al.*, 2001). Accordingly, METH treatment (40 mg/kg; i.p.) induced the release of apoptosis-inducing factor (AIF), Smac/DIABLO and cytochrome *c* from mitochondria into cytoplasmic fractions (Jayanthi *et al.*, 2004). The release of these proteins was followed by activation of caspases 9 and 3 that, in turn, led to the proteolysis of caspase subtracts, such as Poly (ADP-ribose) polymerase (PARP) or lamin A (Jayanthi *et al.*, 2004; Warren *et al.*, 2005, 2007). All together, these events may explain the cell death induced by METH (Jayanthi *et al.*, 2004; Warren *et al.*, 2005, 2007). Furthermore, *in vitro* studies showed that METH-induced apoptosis is due to an early overexpression of Bax, decrease of mitochondrial respiration and membrane potential, and release of mitochondrial cytochrome *c* with subsequent activation of the caspase cascade (Deng *et al.*, 2002). Very recently, our group reported that METH triggers subventricular zone (SVZ) stem/progenitor cell death *via* activation of caspase 3 (Bento *et al.*, 2011). These observations are in agreement with those showing that overexpression of Bcl-2 (Cadet *et al.*, 1997) as well as inhibition of caspases (Uemura *et al.*, 2003; Jimenez *et al.*, 2004) and PARP (Iwashita *et al.*, 2004) can protect against METH-induced cell death.

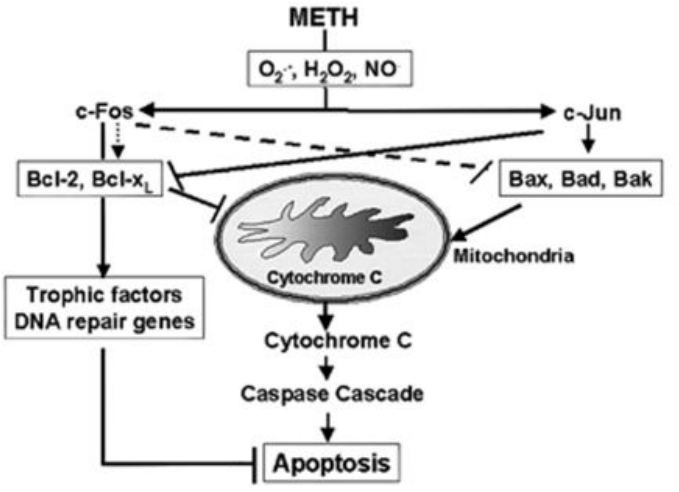


Figure 1.6. *Effect of methamphetamine (METH) on mitochondria. Abbreviations: Bad, Bcl-2-associated death promoter; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2–associated X protein; Bcl-X_L, B-cell lymphoma-extra large; Bcl-2, B-cell lymphoma 2; H_2O_2, hydrogen peroxide; O_2^-, oxide ion; NO, nitric oxide (Cadet et al., 2003).*

1.1.2.5. Blood-brain barrier dysfunction

The above-described effects of METH are usually classified as the classical mechanisms of METH toxicity. However, more recent studies have been focus on the impact of METH on endothelial and glial cells. The mechanisms underlying the negative effect of METH on blood-brain barrier (BBB) are still poorly understood, but some authors have been proposing events related with oxidative stress, hyperthermia and matrix metalloproteinases (MMPs; Silva *et al.*, 2010; Yamamoto *et al.*, 2010). The BBB is a selective barrier responsible for the regulation and maintenance of the brain microenvironment for reliable neuronal function (Abbott *et al.*, 2006, 2010; Weiss *et al.*, 2009), and its dysfunction seems to be involved in several neurological diseases (Weiss *et al.*, 2009), including METH neuropathology (Dietrich, 2009). In fact, it was demonstrated that METH causes marked disruption of BBB in several brain regions like cortex, hippocampus, thalamus, hypothalamus, cerebellum and amygdala (Sharma & Ali, 2006; Kiyatkin *et al.*, 2007; Bowyer *et al.*, 2008; Kiyatkin & Sharma, 2009a; Sharma & Kiyatkin, 2009; Martins *et al.*, 2011).

Recently, our group reported that METH intoxication (30 mg/kg; i.p.) increases the BBB permeability, specifically in the mice hippocampus, involving a decrease in tight-junction (TJ) protein levels, namely claudin-5, occludin and *zonula occludens* 1 (ZO-1), and increases both activity and expression of MMP-9 (Martins *et al.*, 2011). The control of the paracellular transport of molecules through BBB is regulated mainly by TJs (Persidsky *et al.*, 2006), and these proteins can be degraded by gelatinases MMPs causing BBB disruption (Lohmann *et al.*, 2004; Rosenberg, 2009). It was previously described that METH (5-day regimen of 2 mg/kg; s.c.) induced a behavioral sensitization and drug reward, which was accompanied by increase in DA release as well as MMP-2 and -9 activity (Mizoguchi *et al.*, 2007a, 2008b). Moreover, these authors concluded that MMPs can be involved in METH-induced sensitization and reward by regulating extracellular DA levels, since both MMP-2/-9 inhibitors-treated mice and MMP-2/-9 KO mice did not display these behavioral and biochemical effects (Mizoguchi *et al.*, 2007b, 2008b). Furthermore, *in vitro* studies showed that in neuron-astrocyte co-cultures exposed to METH there was the release of MMP-1 and urokinase-type plasminogen activator (uPA), which is an activator of MMPs. Previously, it was discussed that METH leads to oxidative stress, and consequently to ROS production in several brain regions, which, in turn, can also explain BBB dysfunction induced by this drug (Dietrich, 2009). Indeed, *in vitro* studies using human brain microvascular endothelial cells (BMVEC) suggest that METH leads to BBB breakdown *via* negative modulation of TJ proteins complex together with the enhancement of ROS production (Mahajan *et al.*, 2008; Ramirez *et al.*, 2009). Interestingly, the treatment with an antioxidant prevented METH-induced ROS formation, TJs down-regulation and BBB permeability both *in vivo* and *in vitro* (Ramirez *et al.*, 2009). More recently, Park and collaborators (Park *et al.*, 2011) reported that METH-treated immortalized human brain microvascular endothelial cells (hCMEC/D3) show an up-regulation of phosphorylated NADPH-oxidase (NOX) subunit 47, and consequently, an increase in NOX complex activity, which, in turn, leads to production of ROS, alteration of occludin expression and increase

of transendothelial monocyte migration. Moreover, the same study demonstrated that NOX inhibition attenuates METH-induced ROS generation, changes in occludin protein levels, and monocyte migration (Park *et al.*, 2011). Other possible mediator of METH-induced BBB damage is hyperthermia (Kiyatkin & Sharma, 2009b). Bowyer and Ali (Bowyer & Ali, 2006) reported that a single high dose of METH (40 mg/kg; i.p.) increased mice body temperature (> 40.5 °C), which caused a rapid and extensive hippocampal and amydgalar BBB disruption. However, more studies are needed to better understand the mechanisms underlying METH-induced BBB dysfunction.

1.2. Neuroinflammation

In response to injury, such as tissue ischemia, autoimmune responses or infectious agents, human body triggers a host defense response, called inflammation, which locally, within tissues outside the brain, manifests by the classical features of swelling, redness, heat and pain (Allan & Rothwell, 2003; Lucas *et al.*, 2006). For many years, the CNS was considered to be an 'immune privileged' organ, neither susceptible to nor contributing to inflammation (Allan & Rothwell, 2003; Lucas *et al.*, 2006). However, recently, it was revealed that in pathological conditions cells and molecules of the immune system can enter the brain, and some cells of brain parenchyma have the capacity to generate an immune response by themselves (Nguyen *et al.*, 2002). Thus, the inflammatory response in the CNS – neuroinflammation - is a protective mechanism that isolates the damaged brain tissue from uninjured areas, destroys affected cells, and repairs the extracellular matrix (Correale & Villa, 2004). The hallmark of neuroinflammatory process is the activation of microglial cells that are the resident immune effector cells in the brain (Czeh *et al.*, 2011). When activated, these cells generate inflammatory mediators, including pro-inflammatory cytokines, prostaglandins, free radicals and complement, which in turn induce

chemokines and adhesion molecules and recruit immune cells, such as leukocytes (Lucas *et al.*, 2006; Farooqui *et al.*, 2007). Under physiological conditions, these reactions induce a rapid and efficient clearance of pathogens and cell debris from the damaged CNS (Farooqui *et al.*, 2007). However, when uncontrolled it can also lead to the production of neurotoxic factors that may promote several acute and chronic neurogenerative disorders (Glass *et al.*, 2010).

1.2.1. Innate and adaptive immune response

Unlike the systemic organs, the brain immune response against pathogens and cell debris does not rely upon either adaptive immune system (lymphocytes and antibodies) or infiltrating professional phagocytes (neutrophils) to recognize and clear pathogens, but instead the brain developed its own innate immune system based upon the activation of the resident glial cells (Nguyen *et al.*, 2002; Hauwel *et al.*, 2005). The innate immune response must distinguish "non-self" molecules that are not expressed by host cells, designated by pathogen-associated molecular patterns (PAMPs; Medzhitov & Janeway, 2000). Similarly, apoptotic cells express specific molecules called apoptotic cell associated molecular patterns (ACAMPs), which also function as selective "eat me" signals (Hauwel *et al.*, 2005). Then, these PAMPs and ACAMPs are recognized by pattern recognition receptors (PRR), which are innate immune molecules expressed on the surface of resident brain cells, namely Toll-like receptors (TLRs; Gordon, 2002). In fact, the identification of PAMPs by TLRs is considered the first contact between pathogens and the host (Nguyen *et al.*, 2002), and triggers phagocytosis and/or production and release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. In turn, these molecules may facilitate the recruitment and increased activity of other immune players, especially immune cells of adaptive immune system (Simard & Rivest, 2005). So, TLRs signaling is a key mediator between the innate and adaptive immune response in the brain.

In contrast to the fact that innate immune responses frequently emerge within the CNS, it appears more difficult to locally initiate adaptive immune responses (Amor *et al.*, 2010). However, in several neuropathologies, the innate and adaptive immune cells are sequentially activated and mutually regulate each other (Beurel *et al.*, 2010). In fact, an adaptive immune response ensues when the innate immune system encounters a pathogen, recognized by PAMPs, and links the adaptive immune response through antigen presentation of the foreign protein (Stone *et al.*, 2009; Beurel *et al.*, 2010). Then, antigen presenting cells (APC) phagocytes or endocytes foreign pathogens, process and present foreign antigen complexed with surface major histocompatibility complex (MHC) molecules that are recognized by $CD4^+$ (helper) or $CD8^+$ (cytotoxic) T-cells, which become fully activated (Stone *et al.*, 2009). Moreover, activated $CD4^+$ T-cells can, in turn, recruit other T and B cells to sites of inflammation propagating the immune response (Stone *et al.*, 2009). Although naïve T- and B- cells are usually precluded from entry into the CNS, under a neuroinflammatory state, activated glial cells secrete factors, such as cellular adhesion molecules and chemokines, that will disrupt the BBB allowing the entrance of adaptive immune components into the brain (Hisanaga *et al.*, 2001; Babcock *et al.*, 2003).

1.2.2. Immune effectors in the central nervous system

The inflammatory responses in the brain are based on the integrated signaling between neurons, microglia and astrocytes (Bezzi & Volterra, 2001; Allen & Barres, 2009). Recent findings indicated that neurons actively participated in immune regulation in CNS (Biber *et al.*, 2007; de Haas *et al.*, 2007; Levite, 2008; Tian *et al.*, 2009), since they can produce pro-inflammatory cytokines, such as TNF-α or IL-1β (Ravizza *et al.*, 2006; Park & Bowers, 2010), express class I MHC molecules (Foster *et al.*, 2002; Ribic *et al.*, 2010) and suppress T-cell activation (Tian *et al.*, 2009). However, neurons are still the most passive cells in the brain's inflammatory response. Furthermore, a plethora of data have demonstrated that microglia and astrocytes are the two

major cell populations in the CNS responsible for the immune reactions (Hanisch & Kettenmann, 2007; Allen & Barres, 2009; Rossi & Volterra, 2009; Liu *et al.*, 2011).

1.2.2.1. Microglia

Microglia are the main cellular regulators of the brain's innate immune response to both physiological and pathological conditions. These resident immune cells survey the brain for damage and infection, engulfing dead cells and debris. The microglial cells have many features of periphery monocytes and macrophages, since microglial progenitors arise from pial macrophages and mesenchymal tissue, and colonize the CNS during embryonic and fetal periods of development *via* extravascular routes (Chan *et al.*, 2007; Czeh *et al.*, 2011).

In physiological conditions, microglia have a small cellular body and display ramified morphology that is composed of long branching processes, which are constantly moving and surveying the surrounding area (Kim & de Vellis, 2005; Czeh *et al.*, 2011). During this stage, the microglia present also a resting-like down-regulated immune phenotype, because ramified microglia are unable to phagocytize cells and display little or no immunomolecules (Aloisi, 2001; Kim & de Vellis, 2005). Moreover, the purpose of ramified microglia is to maintain a constant level of available microglia to detect and fight infection, while maintaining an immunologically silent environment (Aloisi, 2001). However, resting microglia rapidly modify their morphology and expression of cell surface antigens in response to brain injury (Kim & de Vellis, 2005). In fact, following a brain insult, microglial cells change from a ramified to hyperramified phenotype adopting an amoeboid morphology (Graeber, 2010; Graeber & Streit, 2010; Figure 1.7). Besides their phenotype, activated microglia also proliferate, migrate to the site of damage and secrete pro- and anti-inflammatory cytokines, chemokines, oxidative stress-inducing factors, as well as growth factors (Hanisch & Kettenmann, 2007; Czeh *et al.*, 2011). Moreover, high levels of MHC II, adhesion molecules and phagocytosis receptors are also expressed by microglia (Streit, 2002; Graeber, 2010). In course of recovery from injury,

microglial overactivation is followed by microglial apoptosis, in order to reestablish the normal functionality in the brain (Graeber, 2010).

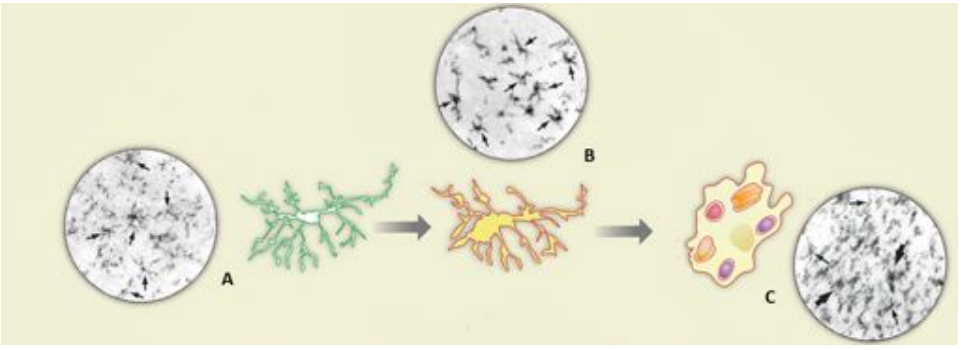


Figure 1.7. *Schematic drawing and representative images showing the microglial activation cascade and associated phenotypic plasticity. (A) Ramified "resting" microglia. (B) Activated microglial cells that still ramified but display stouter cell processes. (C) Activated microglia with amoeboid shape. In all representative* in vivo *images, the microglia was labeled with integrin αM (OX-42) monoclonal antibody (adapted from Graeber, 2010).*

More recently, some authors suggested that 'resting' microglia should be renamed 'surveying' microglia, because these cells actively search for and read signals in the brain environment. Furthermore, they also recommended the change of 'activated' microglial for 'effector' microglia, since it better describes its executor role in response to brain damage (Hanisch & Kettenmann, 2007).

The activation of microglia can have a beneficial or harmful function, and it depends on the type of stress and damage signals, duration/timing of the insult, microenvironment, interaction with other cell types, and age of the organism (Walter & Neumann, 2009). Moreover, it has been shown that microglial activation during stress differs from that during infection (Sugama *et al.*, 2009). In fact, two important signaling principles organize microglial responsiveness that result in different molecular phenotypes and effector functions (Hanisch & Kettenmann, 2007; Czeh *et al.*, 2011). Hence, when microglial cells are challenged by bacterial invasion [i.e. lipopolysaccharide (LPS)], they express cluster of differentiation (CD)86 and CD16/32 and produce high amounts of oxidative metabolites (NO and superoxide), proteases and pro-inflammatory

cytokines, which are essential to host defense against pathogens (Hanisch & Kettenmann, 2007; Czeh *et al.*, 2011). This type of response termed 'classically activated microglia' can also damage healthy cells such as neurons and glial cells (Czeh *et al.*, 2011; Figure 1.8). In contrast, 'alternatively activated microglia' release anti-inflammatory factors and express CD206 and arginase 1 leading to both down-regulation of inflammation and tissue remodeling/repair and angiogenesis (Hanisch & Kettenmann, 2007; Czeh *et al.*, 2011; Figure 1.8). Although microglia have been considered as the first line of brain defense, astrocytes were also recognized as active participants in response to brain injury (Liu *et al.*, 2011). However, the activation of these two effector cells occurs with a spatial and temporal distinct pattern, starting with the activation of microglia followed by astrocyte activation, that, in turn will facilitate the activation of distant microglia (Liu *et al.*, 2011).

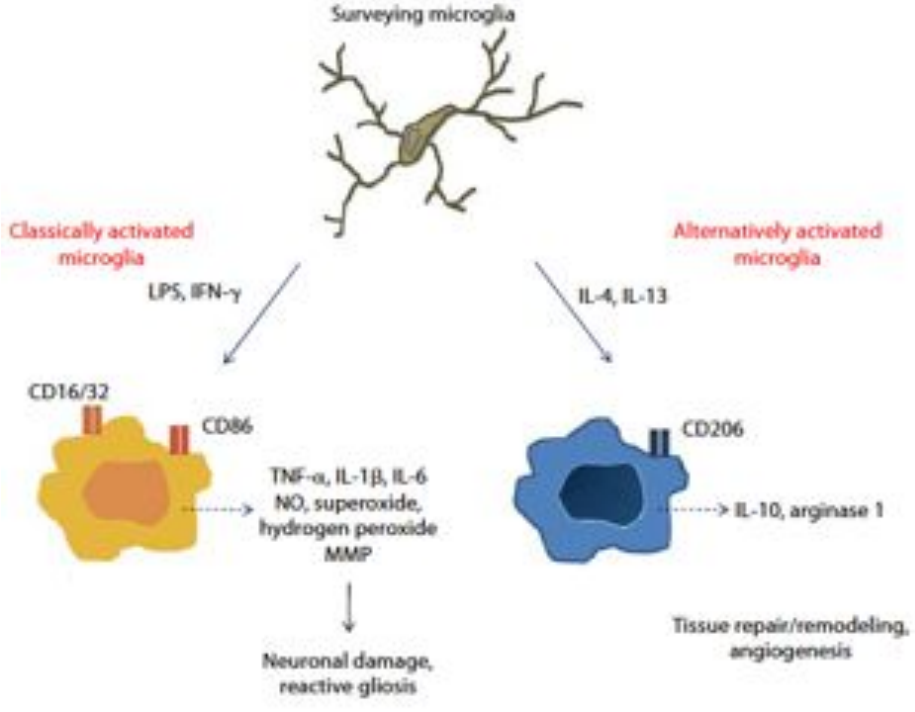


Figure 1.8. *Classically and alternatively activated microglia. The microglial cells can be classified in a simplified manner into two subsets of phenotypes and effector functions depending on the activation pathway (Czeh et al., 2011).*

1.2.2.2. Astrocytes

Astrocytes are the main neural cell type responsible for the maintenance of brain homeostasis (Belanger & Magistretti, 2009). In fact, in healthy CNS, astrocytes provide energy and substrates to neurons, act as physical barriers between the synaptic connections of neighboring neurons, and remove excess neurotransmitter molecules from the extracellular space (Allen & Barres, 2009; Rossi & Volterra, 2009). More recently, these cells have been divided into two main subtypes, protoplasmic and fibrous, on the basis of differences in their cellular morphologies and anatomical locations (Sofroniew & Vinters, 2010). Thus, protoplasmic astrocytes are found in the grey matter and exhibit many branching processes that are intimately associated with neuronal cell bodies and synapses (Allen & Barres, 2009; Sofroniew & Vinters, 2010). On the other hand, fibrous astrocytes are localized in the white matter and have many long fiber-like processes, which are associated with neuronal axons (Allen & Barres, 2009; Sofroniew & Vinters, 2010).

Like microglia, astrocytes can become activated, a process known as astrogliosis, which is characterized by altered gene expression, hypertrophy, and proliferation (Ridet *et al.*, 1997). Indeed, reactive astrocytes release several immune mediators, such as cytokines, chemokines, and growth factors, following brain injury that triggers two types of events as follows (Figure 1.9): one is characterized by the stimulation of neighboring cells by activated astrocytes, amplifying the local and initial immune response; the other occurs when these brain immune cells alter BBB permeability and attract periphery immune cells from the blood vessels (Farina *et al.*, 2007). The changes that undergo during reactive astrogliosis can be beneficial or detrimental to the surrounding neural and non-neural cells (Sofroniew & Vinters, 2010). Actually, numerous studies have reported that reactive astrocytes can protect CNS cells and tissue through multiple ways, including the uptake of glutamate, protection from oxidative stress *via* glutathione production, facilitation of BBB repair or limiting the spread of inflammatory cells or infectious agents (Hamby & Sofroniew, 2010). Nevertheless, it is also clear that activated astrocytes may

play harmful roles during injury or disease through gain of abnormal effects such as over production of ROS or inflammatory cytokines (Hamby & Sofroniew, 2010). Overall, immune activation of astrocytes has the potential to influence injury/disease outcomes both positively and negatively, as determined by specific signaling events and molecular effectors.

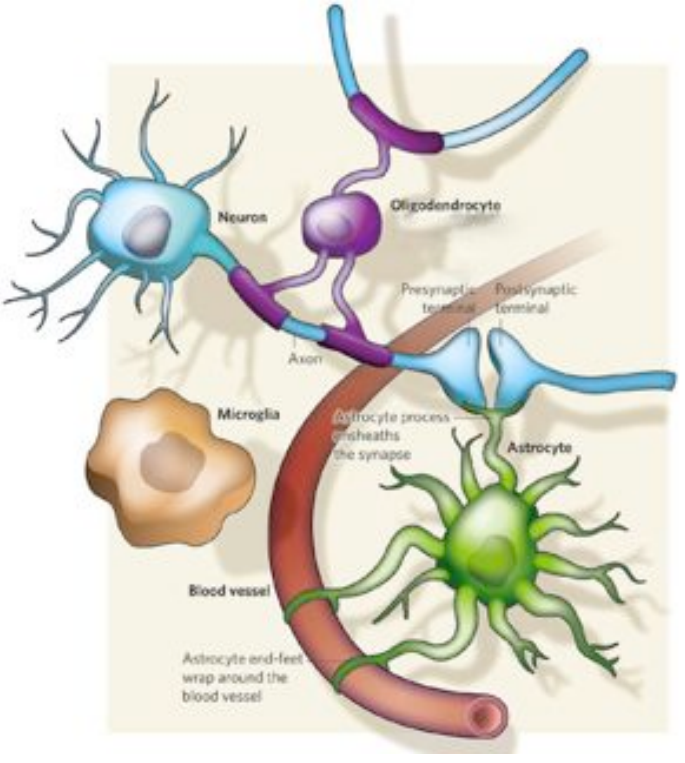


Figure 1.9. *The activation of astrocytes generates waves of innate and adaptive immunity, since their location is strategic allowing the close contact with CNS-resident cells (neurons, microglia, oligodendrocytes and other astrocytes) and with blood vessels (Farina et al., 2007).*

1.2.3. Inflammatory mediators in the brain

In response to injury, infection or disease, resident CNS cells (microglia, astrocytes and neurons) generate inflammatory mediators, namely pro-inflammatory cytokines, which have diverse actions that can trigger, exacerbate and/or inhibit cellular injury and repair (Allan & Rothwell, 2001; Lucas *et al.*, 2006; Farooqui *et al.*, 2007). Although healthy CNS expresses very low, or undetectable, levels of pro-inflammatory cytokines, they are induced rapidly in response to brain damage, and exert diverse actions (Lucas *et al.*, 2006). These

molecules play an essential role in neuroinflammation since they induce chemokines and adhesion molecules release, recruit immune cells into the parenchyma, and activate immune cells and endogenous glial cells (Lucas *et al.*, 2006). There are several pro-inflammatory cytokines, but in the brain the most studied are the TNF-α, IL-1β and IL-6. After an insult, TNF-α and IL-1β are usually the first cytokines to be up-regulated, that in turn, induce the synthesis of IL-6 (Farooqui *et al.*, 2007).

1.2.3.1. Tumor necrosis factor-alpha

TNF-α has been considered the master inflammatory regulator because it can induce further cytokines production, gliosis, BBB damage, demyelination, cell adhesion, and immune reactivity (Kraft *et al.*, 2009). This cytokine is synthesized as a monomeric type-2 transmembrane protein inserted into the membrane as a homotrimer (tmTNF) and cleaved by the MMP TNF-α converting enzyme (TACE), which is a member of the a disintegrin and metalloprotease (ADAM) family, to soluble circulating trimer (solTNF) (MacEwan, 2002; Figiel, 2008; McCoy & Tansey, 2008). Importantly, both tmTNF and solTNF are biologically active and its balance modulates the divergent TNF actions on cellular viability (Gearing *et al.*, 1994; McCoy & Tansey, 2008). Although TNF-α production in the CNS can be attributed to neurons and astrocytes, microglial cells are considered its main source (Hanisch, 2002). In fact, following brain injury, microglia respond with an early, rapid and dramatic up-regulation of this molecule, resulting in a burst of cytokine release (Hanisch, 2002; Kraft *et al.*, 2009). Besides, this prompt production of microglial TNF-α can critically influence subsequent events since low levels of TNF-α are usually neuroprotective, whereas high levels can be neurotoxic (Hanisch, 2002; Bernardino *et al.*, 2005b; Kraft *et al.*, 2009). To date, several studies have reported that TNF-mediated signaling has essential functions within the CNS, including microglial and astrocytes activation, regulation of BBB permeability, febrile responses, glutamatergic transmission,

and synaptic plasticity (Beattie *et al.*, 2002; Hanisch, 2002; Leon, 2002; Pickering *et al.*, 2005; Chaitanya *et al.*, 2011).

The pleiotropic actions of TNF-α are mediated through two distinct cell surface receptors: TNF receptor type 1 (TNFR1 or p55; 55 kDa) and TNF receptor type 2 (TNFR2 or p75; 75 kDa; MacEwan, 2002; Wajant, 2003). Despite the fact that both neurons and glial cells express TNF receptors (Kinouchi *et al.*, 1991), they differ in the expression profiles, ligand affinity and cytoplasmic tail structure (McCoy & Tansey, 2008). Moreover, it seems that these differential patterns of TNF receptors play an important role in determining whether TNF-α will exert a beneficial or harmful effect on CNS (Figiel, 2008). In fact, TNFR1 and TNFR2 influence the activation of several intracellular signaling pathways, including nuclear factor kappa-B (NF-κB), p38 and c-jun N-terminal kinase (JNK), resulting in a number of responses namely inflammation, proliferation, cell migration, apoptosis, and necrosis (Eissner *et al.*, 2004; Ware, 2005; Fenger *et al.*, 2006; Lambertsen *et al.*, 2007; Bernardino *et al.*, 2008). However, it is important to notice that increasing evidence point out to an overlap of their signaling capabilities in mediating biological effects (Declercq *et al.*, 1998; Quintana *et al.*, 2005).

TNFR1 has been extensible studied, in part due to the presence of cytoplasmic death domain in their structure that may directly induce apoptosis (MacEwan, 2002; Figiel, 2008). However, TNFR1 transduction pathway is much more complex because its activation may result in apoptotic events or transcriptional activity (Figure 1.10). Thus, the binding of TNF-α to the extracellular domain of TNFR1 is recognized by the adaptor protein TNF receptor-associated death domain (TRADD), which allows the recruitment of additional proteins specifically the receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2) and FAS-associated death domain (FADD), that are responsible for initiating TNFR1 signaling events (Hsu *et al.*, 1996a; Hsu *et al.*, 1996b). In fact, FADD is able to recruit caspase-8 to the TNFR1 complex and initiates a proteases cascade that leads to apoptosis. On the other hand, TRAF2 can bind to cellular inhibitor of apoptosis protein-1 and -2 (cIAP-1 and

cIAP-2) that are two anti-apoptotic proteins with ubiquitin protein ligase activity. In addition, TRAF2 may also activate a mitogen-activated protein kinase (MAPK) pathway leading to the activation of JNK that, in turn, phosphorylates c-Jun increasing its transcriptional activity. Finally, the protein kinase RIP is critical to the activation of the transcriptional factor NF-κB signaling to initiate pro-survival cascade, cellular proliferation and cytokine production (Hsu *et al*., 1996a, 1996b).

The TNFR2 activation results in fewer biological effects compared to those mediated by TNFR1-dependent signaling, in part due to its restrict expression in the brain and its preference to bind to tmTNF (Figure 1.10; McCoy & Tansey, 2008). Both TNF receptors are quite similar in their extracellular regions, while their intracellular domains exhibit structural differences since TNFR2 does not contain a death domain motif (MacEwan, 2002). Moreover, TNFR2 still recruits adaptor proteins, including TRAF1 and TRAF2, and subsequent activation of NF-κB, MAPK and p38 (MacEwan, 2002; Rauert *et al.*, 2010). So, the principal role of TNFR2 is initiate trophic/protective actions (Kraft *et al.*, 2009), but it can also induce apoptosis by promoting TNFR1 signaling (Tartaglia *et al.*, 1993a). In fact, Tartaglia and collaborators (1993) reported that TNFR2 regulates the rate of TNF-α association with TNFR1 by increasing the local concentration of this cytokine at the cell surface through rapid ligand association and dissociation, which is called ligand passing mechanism. Moreover, the same study suggested that ligand passing mechanism is the primary contribution of TNFR2 to TNF-α-mediated signaling in contrast to direct pathway activation through adaptor protein association within intracellular domain of TNFR2 (Tartaglia *et al.*, 1993a).

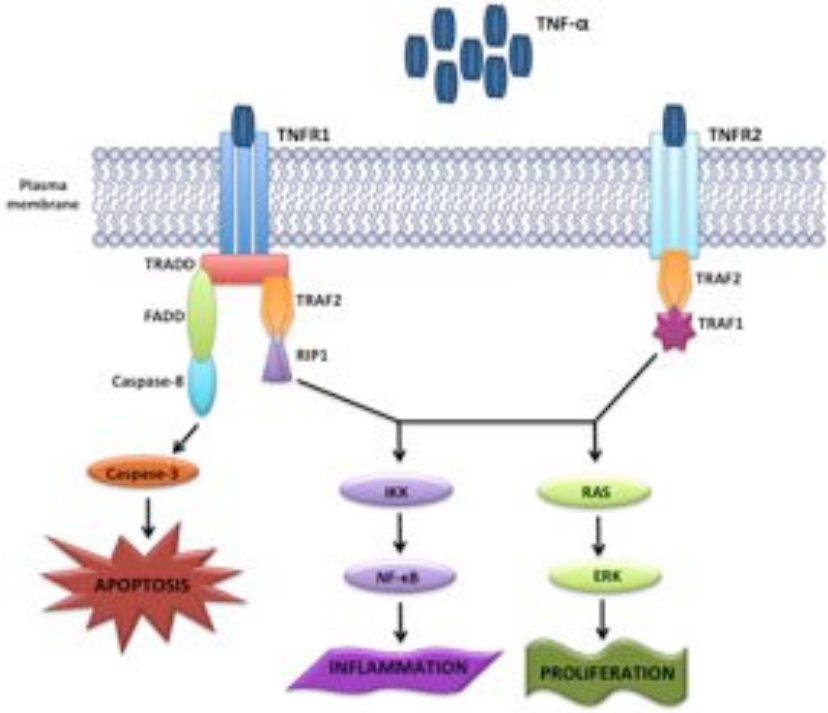


Figure 1.10. *Schematic representation of the signaling pathways activated by the tumor necrosis factor-alpha (TNF-α) receptor type 1 (TNFR1) and 2 (TNFR2). Main abbreviations: ERK, extracellular signal-regulated kinase; IKK, IκB kinase; FADD, FAS-associated death domain; NFκB, nuclear factor kappa-B; RIP1, receptor-interacting protein-1; TNF-α, tumor necrosis factor-alpha; TRADD, TNF receptor-associated death domain; TRAF1, TNF receptor-associated factor 1; TRAF2, TNF receptor-associated factor 2.*

1.2.3.2. Interleukin-1 beta

IL-1 represents a family of proteins critical in orchestrating immune and inflammatory response to injuries and infections. The most extensively characterized member of this family is IL-1β, which is the major secretory form and is reported to have neurotoxic actions (Allan & Rothwell, 2001). In fact, this cytokine upregulates several inflammatory mediators, such as IL-1β itself, TNF-α, cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and chemokines, that are involved in the pathogenesis of acute and chronic neuroinflammation (Basu *et al.*, 2004; Simi *et al.*, 2007). Besides microglial cells, also neurons, astrocytes, oligodendrocytes and endothelial cells may produce

and release IL-1β, but this phenomenon seems to occur only after microglia response (Vitkovic *et al.*, 2000). Similarly to other pro-inflammatory cytokines, within the healthy brain IL-1β is expressed at low levels, which are up-regulated under pathological conditions (Basu *et al.*, 2004). The biosynthesis of IL-1β is complex and is regulated at multiple levels. Thus, this molecule is primarily synthesized as an immature 31 kDa precursor named pro-IL-1β, that in turn is cleaved by the interleukin-1 converting enzyme (ICE or caspase-1) originating the mature form of IL-1β (17.5 kDa).

The IL-1β exerts its actions through the interleukin-1 type I receptor (IL-1RI; 80 kDa). However, there is also the interleukin-1 type II receptor (IL-1RII; 60 kDa), which acts as a decoy receptor since it binds IL-1β without activating signaling (Allan *et al.*, 2005). Nevertheless, to activate the IL-1β pathway *via* IL-1RI is essential the IL-1R accessory protein (IL-1RAcP; 66 kDa) because it increases the binding affinity of IL-1RI for IL-1β allowing the signal transduction (Cullinan *et al.*, 1998). Thus, the binding of IL-1β to IL-1RI causes the recruitment of IL-1RAcP, and this complex requires the intracellular Toll/IL-1R (TIR) domain-containing adapter protein MyD88 that then activates serine/threonine kinases IL-1R-associated kinase (IRAK). Afterwards, IRAK interacts with tumor necrosis receptor associated factor-6 (TRAF6), and several downstream protein kinases are triggered leading to NF-κB and JNK signaling pathway activation, which are involved in survival and inflammatory response (Subramaniam *et al.*, 2004; Figure 1.11). However, IL-1RI can also trigger other cascades, namely p38 and extracellular signal-regulated activated kinase 1 and 2 (ERK1/2; Subramaniam *et al.*, 2004). Additionally, it was demonstrated that the actions of IL-1β strongly induces expression of TNF-α, IL-6 and Cox-2 (Basu *et al.*, 2002).

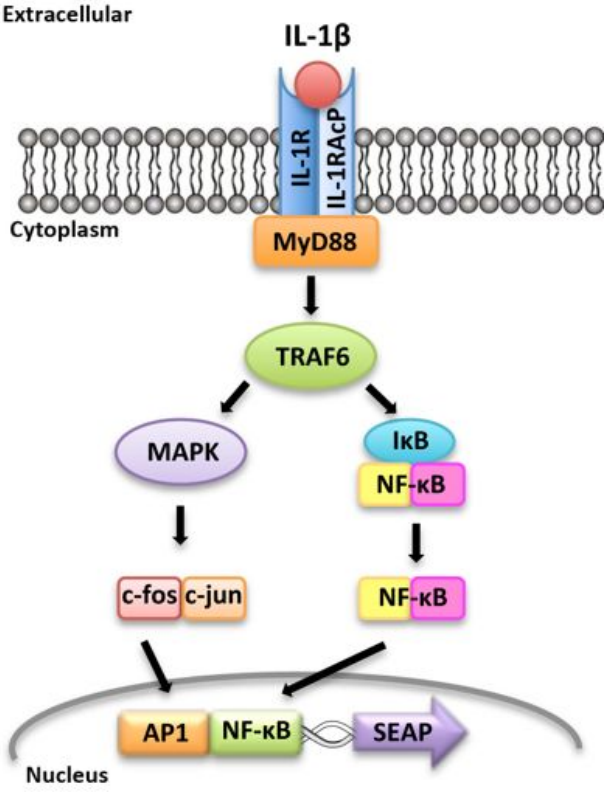


Figure 1.11. *Schematic representation of signaling pathway activated by interleukin-1beta (IL-1β). Main abbreviations: AP-1, activator protein 1; IκB, NF-κB inhibitor; IL-1R, interleukin-1 receptor; IL-1RAcP, IL-1R accessory protein; NF-κB, nuclear factor κ B MAPK, mitogen-associated protein kinase; MyD88, myeloid differentiation primary response gene 88; SEAP, secreted alkaline phosphatase; TRAF-6, tumor necrosis receptor associated factor-6.*

Similarly to TNF-α, IL-1β has a dual role. This pro-inflammatory cytokine has been suggested to be involved in propagation of chronic CNS disease, such as Alzheimer´s and Parkinson's diseases, epilepsy or multiple sclerosis (Basu *et al.*, 2004). On the other hand, IL-1β can exert beneficial effects, particularly when released in modest concentrations, specifically in the survival of neurons and glial cells (Basu *et al.*, 2004)

1.2.3.3. Interleukin-6

IL-6 is a pleiotropic cytokine that belongs to the IL-6-like cytokines family that also includes the IL-11 and leukemia inhibitor factor (LIF). It has pro- and

anti- inflammatory properties (Heinrich *et al.*, 2003) and seems to be involved in the activation of target genes that leads to differentiation, survival, apoptosis and proliferation (Heinrich *et al.*, 2003). Moreover, IL-6 is a glycosylated protein of 21-28 kDa that, in the brain, is produced by neurons and glial cells (Gadient & Otten, 1997; Van Wagoner & Benveniste, 1999).

The biological action of IL-6 is mediated by a receptor complex that consists of type I transmembrane glycoprotein (IL-6R, also designated CD126 or gp80; 80 kDa) and the type I transmembrane signal transducer protein gp130 (CD130; 130 kDa; Scheller *et al.*, 2011). On target cells, IL-6 binds to the membrane-bound IL-6R (mbIL-6R) and then the complex of IL-6/IL-6R binds to two molecules of gp130 leading to IL-6-signal transduction (IL-6 classic signaling; Figure 1.12), which includes activation of JAK/STAT, ERK and PI3K signal transduction pathways (Gadient & Otten, 1997; Heinrich *et al.*, 2003). The gp130 is ubiquitously expressed but the IL-6R is only present in few cell types, namely immune cells (Scheller & Rose-John, 2006). Thus, the cells that only express gp130 developed a signal transduction mechanism called trans-signaling (Figure 1.12; Scheller *et al.*, 2011). Specifically, there is generation of the soluble form of IL-6R (sIL-6R) by ADAM-dependent proteolytic cleavage (shedding) of the membrane-bound IL-6R (mbIL-6R) (Mullberg *et al.*, 1993; Rose-John *et al.*, 1993) or by an alternative splicing of IL-6R mRNA with lack of transmembrane and cytosolic domains (Lust *et al.*, 1992). Then, the sIL-6R binds to IL-6 with comparable affinity as the mbIL-6R, and the IL-6/sIL-6R complex acts agonistically on cells that express gp130 leading to a normal IL-6 signaling pathway (Scheller *et al.*, 2011). Importantly, soluble receptors have been described for many cytokines, such as soluble receptor of TNFR1 (sTNFR1), but in contrast to sIL-6, it inhibits the biologic activity of their ligands acting as antagonist (Rose-John & Heinrich, 1994).

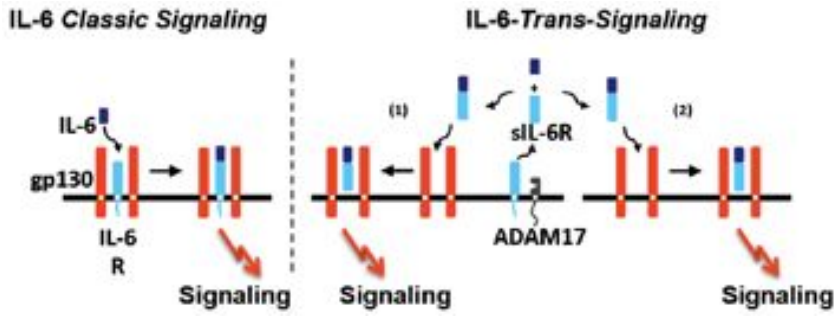


Figure 1.12. *The two types of interleukin-6 (IL-6) signaling activation: the classic and the trans-signaling. Main abbreviations: ADAM17, a disintegrin and metalloproteinase; gp130, type I transmembrane signal transducer protein gp130; IL-6, interleukin-6; IL-6R, type I transmembrane glycoprotein; sIL-6R, soluble form of IL-6R (Scheller et al., 2011).*

Under conditions of brain damage, IL-6 binds to IL-6R that induces homodimerization of gp130 following activation of associated janus kinaes (JAK) (Murakami *et al.*, 1993). Then, JAK activation leads to the tyrosine phosphorylation of signal transducer and activator of transcription (STAT), followed by their dimerization and translocation to the nucleus, where it will bind to elements in the promoters of IL-6 responsive genes (Taga & Kishimoto, 1997). Indeed, it has been demonstrated that, in the CNS, the IL-6 signaling pathway is carried out by STAT3 phosphorylation on tyrosine 705 (Tyr705; Sanz *et al.*, 2008). JAK/STAT is the most important pathway related to IL-6, but this cytokine also activates the MAPK signal transduction pathway (Taga & Kishimoto, 1997). In this case, the activation of gp130/JAK complex recruits another complex formed by Src homology 2-containing tyrosine phosphatase (SHP2)/growth-factor-receptor-bound protein (Grb2)/son of sevenless (SOS), which exchanges GDP to GTP on Ras. Then, this activated kinase triggers the MAPK signaling (Scheller *et al.*, 2011). Thus, both JAK/STAT and MAPK pathways lead to the activation of a number of transcription factors responsible for IL-6 mediated effects (Van Wagoner & Benveniste, 1999) as schematic represented in Figure 1.13.

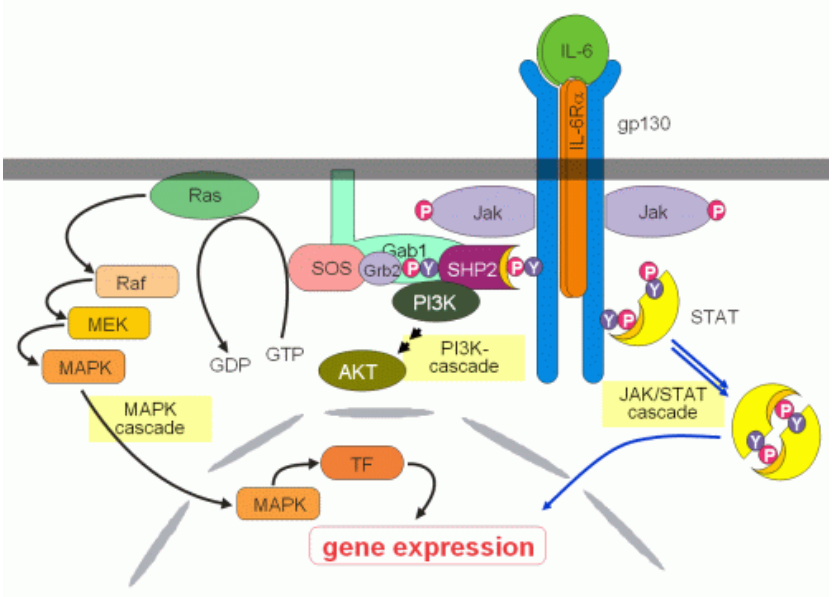


Figure 1.13. *Schematic representation of interleukin-6 (IL-6) induced signal transduction. Main abbreviations: gp130, type I transmembrane signal transducer protein gp130; Grb2, growth-factor-receptor-bound protein; JAK, associated janus kinases; MAPK, mitogen-associated protein kinase; MEK mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; STAT, signal transducer and activator of transcription; SHP2, Src homology 2-containing tyrosine phosphatase; SOS, son of sevenless; TF, transcription factor (Heinrich et al., 2003).*

Several studies have demonstrated that IL-6 may exert multiple beneficial or destructive effects on the CNS. *In vitro* and *in vivo* studies clearly show the IL-6 involvement in neuronal survival, protection, and differentiation (Hirota *et al.*, 1996; Gadient & Otten, 1997; Loddick *et al.*, 1998; Marz *et al.*, 1998). However, IL-6 dysregulation and overexpression also contribute to the neuropathology and pathophysiology associated with many diseases, such as Alzheimer's disease, stroke and traumatic brain injury (Gadient & Otten, 1997).

1.2.4. Neuroinflammation and methamphetamine

As abovementioned the damage of DAergic system, oxidative stress and excitotoxicity are the most widely studied mechanisms involved in METH-induced neurotoxicity. However, recently it was suggested that this drug of abuse could also activate glial cells that through the increase of pro-

inflammatory cytokines production/release contribute to METH-related neuropathology (Cadet & Krasnova, 2009; Silva *et al.*, 2010). In fact, it was reported that chronic METH human abusers display changes in striatal microglia and astrocytes (Kitamura *et al.*, 2010). Furthermore, Loftis *et al*. (2011) verified an up-regulation of cytokines, especially interferon-γ (IFN-γ), TNF-α, IL-1β and IL-6, as well as chemokines, namely monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) and -1β, in human plasma of METH users when compared with healthy subjects. Additionally, the authors also showed that these changes in inflammatory molecules were accompanied by deficits in learning and memory and in attention/information processing (Loftis *et al.*, 2011).

In addition to human studies, many other studies using animal models support the hypothesis of a critical role of neuroinflammation in METH-induced toxicity. Indeed, it was reported that METH (20 mg/kg, s.c.) induced an up-regulation of IL-1α, TNF-α and IL-6 mRNA levels together with an increase of chemokine MCP-1 expression in mouse striatum (Sriram *et al.*, 2006b). Accordingly, an *in vitro* study showed that METH leads to an up-regulation of IL-1β, TNF-α and IL-6 mRNA, as well as formation of ROS and RNS in rat microglial cell line (HAPI; Tocharus *et al.*, 2010). TNF-α release/production seems to be an important feature of METH-induced neuroinflammation. In fact, a repeated METH treatment (2 mg/kg/day for 5 days; s.c.) leads to TNF-α overexpression in the rat brain (Nakajima *et al.*, 2004). Furthermore, the authors verified that mice pre-treated with exogenous TNF-α (4 μg) attenuated METH-induced increase and decrease of extracellular striatal DA and vesicular DA uptake, respectively (Nakajima *et al.*, 2004). Taken together, these findings strongly suggest that TNF-α plays a neuroprotective role in METH-induced drug dependence and neurotoxicity (Nakajima *et al.*, 2004). Concerning IL-1β, there are studies reporting that 15 mg/kg and 45 mg/kg METH (i.p.) causes a marked induction of its mRNA in rat (Yamaguchi *et al.*, 1991) and mice (Numachi *et al.*, 2007) hypothalamus, respectively. Interestingly, Numachi et al. (2007)

concluded that DAT/SERT double knockout (KO) mice injected with 45 mg/kg METH showed neither IL-1β expression augment nor hyperthermia. So, the authors postulated that these findings were, in part, the explanation for the mechanism of METH-induced hyperthermia, since IL-1β is known to be an endogenous pyrogen (Leon, 2002). IL-6 has been described as dual agent in several neuropathology (Scheller *et al.*, 2011), but the precise role of this cytokine under METH neurotoxicity remains unknown. Indeed, (Ladenheim *et al.*, 2000) showed that IL-6 KO was protected from depletion of DA and 5-HT terminals in caudate-putamen and frontal cortex, as well as cortical reactive gliosis and apoptosis induced by repeated administration of METH (5 or 10 mg/kg x 4, 2h intervals; i.p.) On the other hand, other authors demonstrated that IL-6 is required for a cytotropic response to support sprouting of dopaminergic neurons (Liberto *et al.*, 2004). The results of the studies showing evidence of neuroinflammatory process following METH administration are summarized in Table 1.1.

1.2.5. Use of anti-inflammatory drugs as a therapeutic approach

To date, the most effective intervention to treat METH addiction is a detoxification followed by a behavioral therapy. However, nowadays it was hypothesized that anti-inflammatory drugs could be a pharmacological strategy to reduce METH neurotoxicity. This idea is due to the fact that neuroinflammatory mediators seem to be key players in the toxicity induced by METH, which in turn can be related with cyclooxygenase (COX) induction (Minghetti, 2004). Furthermore, neuroinflammation associated with COX production has been implicated in several deleterious events (e.g. pro-inflammatory activity, oxidative stress) resulting in neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Minghetti, 2004). In agreement, it was shown that METH (4 mg/kg x 4, 2h intervals; s.c.) increased striatal COX-2, but not COX-1 protein levels (Kita *et al.*, 2000; Thomas & Kuhn, 2005a). Hence, it is reasonable to suggest that COX-2 is a requisite factor in METH-induced

neurotoxicity (Kita *et al.*, 2000; Thomas & Kuhn, 2005a; Zhang *et al.*, 2007). Recently, a case report described that the administration of minocycline (100 mg/day, 2 times a day, morning and evening), which is a tetracycline-related antibiotic with anti-inflammatory (Tikka *et al.*, 2001) and anti-apoptotic properties (Wang *et al.*, 2004), gradually improved METH-related psychotic symptoms, including hallucinations, social withdrawal and aggressive behaviors, in a Japanese female patient (Tanibuchi *et al.*, 2010). In accordance, mice pre-treated with minocycline (40 mg/kg; s.c.) showed improvement in several features of METH toxicity (Zhang *et al.*, 2006). Specifically, the authors verified that minocycline administration ameliorates the acute hyperlocomotion, the development of behavioral sensitization and the dopaminergic terminals apoptosis induced by a single high dose (3 mg/kg; s.c.), a chronic treatment (1 mg/kg for 5 day; s.c.) and a repeated administration (3 mg/kg, 3h intervals; s.c.) of METH (Zhang *et al.*, 2006). Additionally, (Mizoguchi *et al.*, 2008a) observed that both minocycline post- and co-treatment (40 mg/kg; i.p.) ameliorated the impairment of recognition memory, but had little effect on the behavioral sensitization followed by the chronic administration of METH (1 mg/kg, once daily for 7 days; s.c.) in mice. On the contrary, other authors demonstrated that minocycline (10 mg/kg or 100 mg/kg, s.c.) failed to protect against METH-mediated loss of striatal TH and DA, as well as to modulated TNF signaling (Sriram *et al.*, 2006b). Although minocycline diminished the production of COX in neuroinflammatory process (Ryu *et al.*, 2004), this tetracycline compound is not a selective COX inhibitor. So, several authors had evaluated the role of non-steroidal anti-inflammatory drugs (NSAID) under METH neurotoxicity. It was reported that both ketoprofen (2 or 5 mg/kg x 4; s.c.) and ibuprofen (10 or 20 mg/kg x 4; s.c.) given 30 min prior to each METH injection (4 mg/kg x 4, 2h intervals; i.p.) attenuated mice striatal dopamine terminal degeneration, COX-2 expression and microgliosis (Asanuma *et al.*, 2003; Tsuji *et al.*, 2009). On other hand, pre-treatment with celecoxib (7,5 mg/kg; i.p.) exacerbated striatal DA depletion induced by METH (5 mg/kg x 4, 2h intervals; i.p.; Zhang *et al.*, 2007).

Overall, neuroinflammatory process seems to have an important role in METH-induced neuropathology. In fact, the treatment with anti-inflammatory drugs has protective effects against METH neurodegeneration, which suggests an association between neuroinflammation and brain damage. However, it remains unclear whether the inflammation is the cause or is simply a consequence of the toxicity induced by this drug.

Table 1.1. *Summary of studies showing that METH induces neuroinflammation (Silva et al., 2010)*

Dose Scheme	Strain/Specie	Major findings	Reference
15 mg/kg x4, 2h intervals; s.c.	Sprague-Dawley rats	• Microglial activation • Pathological changes in striatal DA fibers	LaVoie *et al.*, 2004
15 mg/kg; i.p.	Sprague-Dawley rats	• ⇑ IL-1β mRNA in the hypothalamus	Yamaguchi *et al.*, 1991
20 mg/kg; s.c.	B6.129S mice C57BL/6J mice	• ⇑ F4/80, IL-1α, IL-6, CCl2 and TNF-α mRNA in the striatum • Minocycline ⇓ drug-mediated microglial activation, but failed to afford neuroprotection	Sriram *et al.*, 2006b
2 mg/kg/day, 5 days; s.c. 4 mg/kg, 2h intervals; s.c.	Wistar rats C57BL/6J mice	• ⇑ TNF-α protein levels in the nucleus accumbens • Exogenous TNF-α blocked METH-induced hyperlocomotion, rewarding effects and DAergic neurotoxicicty in striatum	Nakajima *et al.*, 2004
45 mg/kg; i.p.	Combination of C57BL/6J and 129v/J mice	• ⇑ IL-1β mRNA in the hypothalamus	Numachi *et al.*, 2007
5 or 10 mg/kg x4, 2h intervals; i.p.	C57BL/6J mice	• METH neurotoxicity is attenuated in IL-6$^{-/-}$ mice	Ladenheim *et al.*, 2000
4 mg/kg x4, 2h intervals; s.c.	BALB/c mice	• ⇑ COX-2 protein expression • ⇓ DA levels in striatum	Kita *et al.*, 2000

Table I.1. *(cont.). Summary of studies showing that METH induces neuroinflammation (Silva et al., 2010)*

Dose Scheme	Strain/Specie	Major findings	Refrences
5 mg/kg x4, 2h intervals; i.p.	BALB/cAnNCrlCrlj mice	• Minocycline ⇓ METH-induced hyperlocomotion, behavioral sensitization, microgliosis, ⇓ DA and DOPAC levels and DAT immunoreactivity in the striatum	Zhang *et al*., 2006
5 mg/kg x4, 2h intervals; i.p.	BALB/c mice	• Ibuproben ⇓ METH-induced reduction of DAT-positive signals and accumulation of activated microglial cells in the striatum	Tsuji *et al*., 2009
5 mg/kg x4, 2h intervals; i.p.	BALB/c mice	• Ketoproben ⇓ METH-induced neurotoxicity and microgliosis in the striatum	Asanuma *et al*., 2003
5 mg/kg x4, 2h intervals; i.p.	Sprague-Dawley rats	• ⇑ Apoptotic cells • ⇓ DA content, TH and MAP2 immureactivity; • Celecoxib ⇑ METH-induced DA depletion in the striatum	Zhang *et al*., 2007
5 mg/kg x4, 2h intervals; i.p.	C57BL/6J	• ⇑ COX-2 protein expression in the striatum • Genetic inactivation of COX-2 is protective against drug-induced neurotoxicity	Thomas & Kuhn, 2005a

1.3. Neuropeptide Y

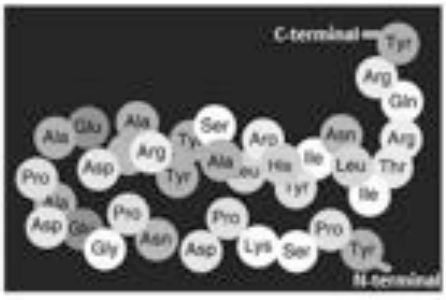
The neuropeptide Y (NPY) belongs to the neuropeptide tyrosine family, which also includes peptide YY (PYY), pancreatic polypeptide (PP) and non-mammalian pancreatic peptide Y (PY) (Michel *et al.*, 1998). These peptides are evolutionarily ancient and structurally conserved. They are also involved in the regulation of fundamental cellular processes of growth, metabolism and cell-cell communication, as well as in the orchestration of body's integrated responses to ensure survival and adaptation to the changing demands of the internal and external environment (Larhammar, 1996).

In mammals, NPY exists in both central and peripheral nervous systems (Pedrazzini *et al.*, 2003). PYY is mainly produced in gut endocrine cells and released following food intake, contributing to the inhibition of gut motility and pancreatic secretion (Fujimiya & Inui, 2000; Naruse *et al.*, 2002). More recently, PYY was found to reach the hypothalamus and reduces appetite (Batterham *et al.*, 2002). Similar with PYY, PP reduces pancreatic secretion, gut motility and appetite, but is released by pancreatic F cells (Hazelwood, 1993; Batterham *et al.*, 2003). Thus, within the same peptide family, there are two peptides that evolve very slowly - NPY and PYY - whereas the third - PP - had an unusual high rate of evolutionary changes (Larhammar, 1996). Moreover, NPY has neurotransmitter properties (Lundberg, 1996), whereas PYY and PP act as endo- and exocrine hormones (Vona-Davis & McFadden, 2007).

1.3.1. NPY structure

The NPY, as well as the other members of NPY family, are composed of 36 amino acids and share considerable homology, amidated carboxy-terminal (C-terminal) ends and the presence of large number of tyrosine (Figure 1.14). Furthermore, the presence of the C-terminal amidation seems to be a protective strategy against carboxypeptidases, which is a characteristic shared by many biologically active peptides (Ludwig *et al.*, 1996). Importantly, the NPY family peptides exhibit a characteristic amino acid residues necessary to adopt

a specific three-dimensional structure that has been termed the pancreatic polypeptide fold (PP-fold) (Blundell *et al.*, 1981). The PP-fold is a tertiary structure with U-shape and consists of an extended polyproline helix following an α-helix connected by a β-turn (Michel *et al.*, 1998).




```
                1         10        20        30   36
                |         |         |         |    |
NPY             YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-amide
                ** **  *****  *  ***  **** ** *****
PYY             YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-amide
                 *  *  **  *  * ** *   ** *** **  **
PP              APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-amide
```

Figure 1.14. *PP-fold structure of neuropeptide Y (NPY) molecule with the hairpin loop structure characteristic of the family of NPY related peptides (adapted from http://flylib.com/books/en/4.85.1.23/1/). Alignment of human NPY, peptide YY (PYY) and pancreatic polypeptide (PP); amino acid identities between peptides are indicated by * (Michel et al., 1998).*

Although PP was discovered first than NPY, evolutionary analysis shows that PP is actually the newest member of the family (Larhammar, 1996). Both NPY and PYY are found in representatives of all major vertebrates groups. In fact, seven positions are constant among all species of NPY, PYY and PP: Pro^{5}, Pro^{8}, Gly^{9}, Ala^{12}, Tyr^{27}, Arg^{33} and Arg^{35} (Figure 1.14). Moreover, highly conversed positions are Pro^{2}, Tyr^{20}, Thr^{32} and Tyr^{36} (Cabrele & Beck-Sickinger, 2000).

1.3.2. NPY biosynthesis

The NPY gene is located on human chromosome 7 at the locus 7p15.1 (Cerda-Reverter & Larhammar, 2000). Similar to the other NPY-related

peptides, this peptide is synthesized as a 97 amino acid peptide precursor that is composed by four exons (Minth *et al.*, 1984). Moreover, the release of mature/active peptide involve the action of endoproteolytic enzymes, which are able to flank the peptide sequence at cleavage sites (Beck-Sickinger & Jung, 1995). The pre-pro-NPY, a peptide precursor with 97 amino acids, following its generation is directed into the endoplasmic reticulum where a hydrophobic signal peptide (28 amino acids) is removed and pro-NPY produced (Figure 1.15). The precursor pro-NPY (69 amino acid) is cleavage at a dibasic site by prohormone convertases generating NPY(1-39) and C-flanking peptide of NPY (CPON; Figure 1.15). Then, a truncation at the C-terminal end by a carboxypeptidase originates the NPY (1-37), which is a substrate for the enzyme peptidylglycine alpha-amidating monooxygenase and leads to the biologically active amidated NPY(1-36) (Figure 1.15). The amide moiety is essential for the activity of NPY and prevents degradation by carboxypeptidases. Furthermore, two enzymes, the dipeptidyl peptidase IV (DPP-4) and the aminopeptidase P, can process the mature NPY resulting in NPY(2-36) and NPY(3-36) (Mentlein *et al.*, 1993; Medeiros & Turner, 1994). This process is crucial since it may regulate the biological activity of NPY by influencing their binding to different receptor subtypes (Mentlein *et al.*, 1993; Medeiros & Turner, 1994).

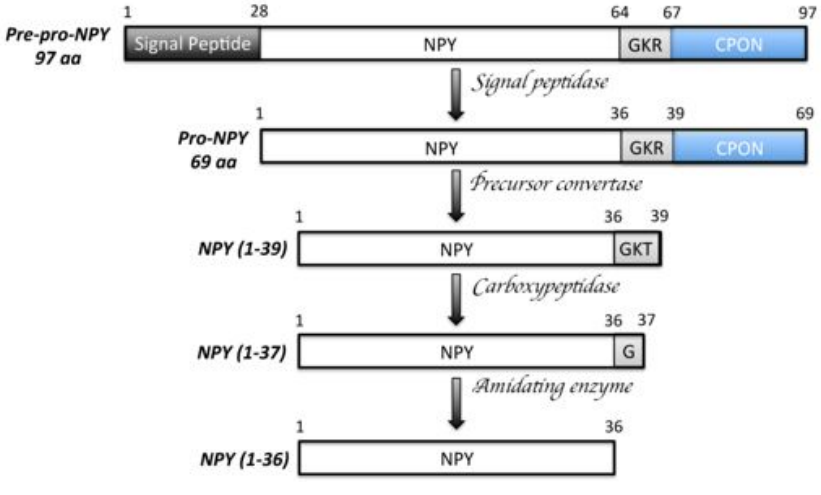


Figure 1.15. *Schematic biosynthesis of NPY. Main abbreviations: CPON, C-flanking peptide of NPY; GKR, Glycine, Lysine, Arginine (adapted from Silva et al., 2002).*

1.3.3. NPY distribution

1.3.3.1. In the peripheral nervous system

NPY is one of the most abundant and widely distributed neuropeptides in both peripheral nervous system (PNS) and CNS. Indeed, in sympathetic neurons, NPY is stored and released together with tyrosine hydroxylase (TH), norepinephrine (NE) and dopamine-β-hydroxylase (DBH), suggesting that NPY-positive nerves are noradrenergic (Lundberg *et al.*, 1983, 1990). Over the years many studies have been revealing the distribution of NPY in the peripheral organs. NPY-immunoreactive fibers in sympathetic ganglia as well as in tissue with sympathetic innervation have already been identified (Lundberg *et al.*, 1983). Moreover, NPY is expressed in the gastrointestinal tract, pancreas, liver, salivary gland, urogenital system and airways (Sundler *et al.*, 1989).

1.3.3.2. In the central nervous system

In the mammalian CNS, NPY has been found in two types of neurons: interneurons and long projection cells (Chronwall *et al.*, 1985; de Quidt & Emson, 1986a, 1986b). Several studies demonstrated that NPY-immunoreactivity (NPY-ir) is abundantly found in many rat brain areas, namely hypothalamus, *nucleus accumbens*, *septum* and *locus coeruleus*. Furthermore, moderated levels are also found in the hippocampus, cerebral cortex, basal ganglia and thalamus (Allen *et al.*, 1983; de Quidt & Emson, 1986a, 1986b).

In cerebral cortex of adult rodent, NPY is expressed within 1-2% of all neuronal population (Aoki & Pickel, 1989) and its immunoreactivity is present in almost all nicotinamide adenine dinucleotide phosphate (NADPH-d), as well as somatostatin (SOM) positive neurons (Papadopoulos *et al.*, 1987; Huh *et al.*, 1997). Furthermore, it has been shown that nearly all NPY-ir neurons are also immunoreactive for γ-aminobutyric acid (GABA) and for glutamic acid decarboxylase (GAD) (Papadopoulos *et al.*, 1987; Aoki & Pickel, 1990). Cortical NPY cells are interneurons and co-exists with GABA, which suggest that these cells are mainly inhibitory interneurons (Aoki & Pickel, 1990). Regarding human cerebral cortex, the amount of NPY-positive cells differs between cortical areas,

being the cingulated and temporal cortical areas that express the highest levels, whereas the occipital lobe the lowest (Adrian *et al.*, 1983). Similar to rodents, the NPY-ir in human cerebral cortex co-localized with NADPH- and SOM-positive neurons (Schwartzberg *et al.*, 1990; Kunzle & Unger, 1992). Besides, it was also verified that all cortical NPY-positive neurons express the glutamate receptor subunit GluR2/4 and, in contrast, only about 17 and 16% them express GluR2 and GluR5/6/7 subunits, respectively (Gonzalez-Albo *et al.*, 2001).

There is a wide distribution of NPY-ir and mRNA in the hippocampus of different species, including rodents and humans (Chronwall *et al.*, 1985; de Quidt & Emson, 1986a, 1986b; Caberlotto *et al.*, 2000). In rodent brain, NPY-positive cells are found in the hilus of the dentate gyrus (DG), stratus oriens of *cornu ammonis* 2 (CA2) and *cornu ammonis* 3 (CA3) subfields and also inside and around the stratum pyramidale of the *cornu ammonis* 1 (CA1) sub-region (Chronwall *et al.*, 1985; de Quidt & Emson, 1986a, 1986b). In addition, hippocampal NPY-ir neurons have few or no α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunits (McDonald, 1996). In human hippocampus, high densities of NPY-ir and mRNA are found in neurons of the dentate hilus, the CA1 and the subicular complex (Chan-Palay *et al.*, 1986; Caberlotto *et al.*, 2000).

One of the brain regions that contain the highest levels of NPY is the striatum (de Quidt & Emson, 1986a, 1986b). In fact, it was observed that *caudate nucleus*, *putamen* and *nucleus accumbens* show a high number of NPY-positive neurons (Adrian *et al.*, 1983; Allen *et al.*, 1983). Furthermore, in this brain region, the NPY-expressing neurons totally co-localized with SOM- and NADPH- positive cells and have a partial co-existence with GABA neurons (Aoki & Pickel, 1989). Besides, some striatal aspartate-positive neurons show NPY-ir (Pettersson *et al.*, 1996).

Despite the high levels of NPY in neurons, recent studies demonstrated that its expression is not restricted to these cells being microglial cells also a source of NPY (Barnea *et al.*, 2001; Ferreira *et al.*, 2010).

1.3.4. NPY receptors

The NPY family members exert their actions *via* a large super-family of G-protein coupled receptors (GPCRs; Michel *et al.*, 1998). In fact, the typical signaling response of NPY receptors leads to inhibition of adenylate cyclase (AC), which indicates that NPY receptors act through pertussis toxin-sensitive G-proteins, e.g., members of the G_i/G_o family (Michel *et al.*, 1998). The NPY receptors are designated by a capital Y, because the members of NPY family contain many tyrosine residues, which are abbreviated by the letter Y. Thus, until now were described 6 subtypes of NPY receptors: Y_1, Y_2, Y_3, Y_5 and y_6 (Figure 1.16). To date, five distinct NPY receptors have been cloned and sequence comparisons show that Y_1, Y_4 and y_6 are more closely related to each other than to the receptors Y_2 and Y_5 (Larhammar, 1996). Moreover, the Y_1, Y_2 and Y_5 receptors preferentially bind NPY and PYY and, in contrast, Y_4 is preferentially activated by PP. Although y_6 receptor has been cloned, its function is unknown because encodes for a truncated non-functional receptor in human (Michel *et al.*, 1998). Concerning Y_3 receptor, it has not been cloned and no specific agonist or antagonist have been described yet (Lee & Miller, 1998).

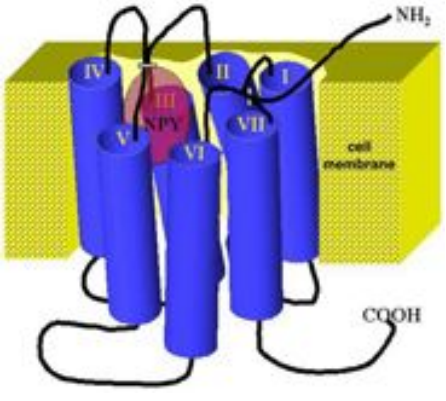


Figure 1.16. *Scheme of NPY receptors, showing the structural characteristics of the seven transmembrane domain-G-protein-coupled receptors (adapted from http://people.usd.edu/).*

1.3.4.1. Y_1 receptor

The Y_1 receptor was the first PP-fold peptide binding receptor to be cloned from rat complementary deoxyribonucleic acid (cDNA; Eva *et al.*, 1990) and

exhibits a highly conserved structure with overall identities of 94% or higher between mammals (Larhammar *et al.*, 2001). To become fully activated, this receptor requires intact N and C termini of NPY (Nakamura *et al.*, 1995). In fact, the mammalian Y_1 receptor does not discriminate between NPY, PYY or peptides modified on C-terminal end; however, truncation of NPY leading to NPY(2-36), NPY(3-36) or NPY(13-36) results in marked loss of affinity and biological activity (Larhammar *et al.*, 1992). The first selective agonist of Y_1 receptor to be described was [Leu^{31},Pro^{34}]NPY, and was synthesized by combining the hexapeptide of PP with the PP-fold of NPY, which allows the replacing of Ile^{31} and Gln^{34} of porcine NPY by Leu^{31} and Pro^{34} of human PP (Fuhlendorff *et al.*, 1990). This agonist was found to be a specific high-affinity ligand for Y_1 receptors and react very poorly with Y_2 receptors (Fuhlendorff *et al.*, 1990), which was helpful in distinguishing between these two subtypes of NPY receptors. Moreover, it was proposed that upon agonist stimulation occur rapid internalization of Y_1 receptors *via* the clathrin-coated pits recycling pathway (Parker *et al.*, 2001; Gicquiaux *et al.*, 2002). Concerning antagonists, the non-peptide BIBP3226 has been defined as a high potency antagonist of Y_1 receptors, and its affinity to other NPY receptors subtypes exceeds 10 μm (Wieland *et al.*, 1995). Moreover, two other antagonists are also widely used: LY357897 (Hipskind *et al.*, 1997) and BIBO3304 (Wieland *et al.*, 1998).

Several studies demonstrated that the Y_1 receptor is detected in a variety of tissues including brain, heart, kidneys and gastrointestinal tract. In the mammalian brain, Y_1 mRNA is expressed at high levels in the cerebral cortex, hippocampus, thalamus and hypothalamus (Kishi *et al.*, 2005). Furthermore, within rodent hippocampus, CA3 and CA1 sub-regions show moderate levels of Y_1 receptor mRNA while higher levels of this receptor are present in DG layer (Kishi *et al.*, 2005). In agreement, *in situ* hybridization experiments in human hippocampus revealed very high levels of Y_1 in the DG when compared to CA3 and CA1 (Caberlotto *et al.*, 1997). Additionally, a lack of co-localization between Y_1 receptor- and NPY-positive neurons in both human and rodent brain suggests that this subtype of receptor is mainly postsynaptic (terminal, dendritic, and/or

somatic localization) (Caberlotto *et al.*, 2000; Kopp *et al.*, 2002). However, immunohistochemical studies identified the presence of Y_1 receptor in NPY-ir terminals of the rat *nucleus accumbens* and cultured rat hippocampal neurons, which indicates that these receptors may also be presynaptic and may act as autoreceptors to regulate NPY release (Pickel *et al.*, 1998; St-Pierre *et al.*, 1998).

The most important Y_1-mediated physiological role of NPY in the brain are related with food intake (Gehlert, 1999), anxiety/depression (Wu *et al.*, 2011) and ethanol dependence (Schroeder *et al.*, 2003).

1.3.4.2. Y_2 receptor

The cDNA of Y_2 receptor was first cloned from human SMS-KAN cells (neuroblastoma cell line) (Rose *et al.*, 1995) and afterwards from human brain cDNA libraries (Gehlert *et al.*, 1996). This receptor subtype is pharmacologically characterized by high affinity to NPY and PYY but, unlike the Y_1 receptor, is relatively resistant to the N-terminal deletion and retains a high binding affinity to the C-terminal fragments of NPY e.g. NPY(2-36), NPY(3-36) and NPY(13-36) (Michel *et al.*, 1998). Moreover, the Y_2 receptor does not appear to be internalized after prolonged agonist stimulation or does so very slowly (Parker *et al.*, 2001). The first antagonist created for Y_2 receptor was T_4-[NPY(33-36)] (Grouzmann *et al.*, 1997), followed by the non-peptide BIIE0246 (Doods *et al.*, 1999). More recently, it was synthesized a selective brain penetrant small molecule antagonist of the Y_2 receptor, JNJ-31020028; however, its characterization requires more studies (Shoblock *et al.*, 2010; Swanson *et al.*, 2011).

In mammals, Y_2 receptor is highly conserved between species with identity around 90% (Larhammar *et al.*, 2001). Indeed, it was demonstrated that Y_2 receptor mRNA is discretely localized in the rat and mouse brain, namely in the hippocampus, hypothalamus, thalamus, amygdala and brainstem (Dumont *et al.*, 1998; Naveilhan *et al.*, 1998). Moreover, the highest levels of mRNA of this receptor in hippocampal formation are found in the CA3 pyramidal cell layer (Naveilhan *et al.*, 1998). Regarding human brain, Y_2 receptor mRNA is present in

the different cerebral cortical areas, the subcortical white matter, striatum and amygdaloid complex (Caberlotto *et al.*, 1998b). Besides, the mRNA of this NPY receptor subtype is also found in the hippocampal formation and, like rodent brain, shows very high levels in the CA3 sub-field (Caberlotto *et al.*, 1998b). In general, Y_2 receptor expression levels are lower when compared to Y_1 subtype (Parker & Herzog, 1999). The co-localization of Y_2 receptor with NPY in neuronal cells of human brain regions (Caberlotto *et al.*, 2000) supports the theory of a presynaptic localization where it acts as an autoreceptor, inhibiting the release of NPY and other neurotransmitters (King *et al.*, 1999; Silva *et al.*, 2001).

In the CNS, the activation of Y_2 receptor by NPY is implicated in several physiological functions, such as the control of food intake (Edelsbrunner *et al.*, 2009), circadian rhythmus (Soscia & Harrington, 2005), anxiety (Wu *et al.*, 2011), neuronal excitability and epilepsy (Silva *et al.*, 2005b; Xapelli *et al.*, 2007), alcohol dependence (Thorsell *et al.*, 2002), cognitive processes (Redrobe *et al.*, 2004) and locomotor activity (Edelsbrunner *et al.*, 2009).

1.3.4.3. Y_3 receptor

The Y_3 receptor is the only NPY receptor subtype that has not been cloned and no selective agonists and antagonists have been described (Michel *et al.*, 1998). However, it is known that this receptor display affinity for the C-terminal fragment, as the Y_2 subtype, but it is insensitive to PYY (Michel *et al.*, 1998).

Few studies described the presence of Y_3 receptor subtype but there are some tissues that express this receptor, such as the human adrenal medulla, rat superior cervical ganglia sympathetic neurons, rat *nucleus tractus solitaries*, rat cardiac ventricular membranes and rat distal colon (Lee & Miller, 1998; Cavadas *et al.*, 2001). Regarding the CNS, once again little is known about the presence and distribution of this NPY receptor subtype, but it was reported that a putative Y_3 receptor is expressed in CA3 sub-region of the hippocampus (Monnet *et al.*, 1992), and electrophysiological studies revealed its presence in the brainstem (Glaum *et al.*, 1997). Furthermore, these receptors have been found in a group of neurons in the *nucleus tractus solitarius* suggesting the

involvement of Y_3 receptor in bradychardia, hypotension and blockade of the local effects of glutamate (Grundemar *et al.*, 1991).

1.3.4.4. Y_4 receptor

The gene for Y_4 receptors was initially cloned from a human genomic library and the receptor derived from this clone was originally designated "PP1", because of its preference for PP as a ligand (Lundell *et al.*, 1995). In fact, this receptor subtype shows more affinity to PP than to NPY or PYY (Michel *et al.*, 1998). Moreover, the Y_1 receptors antagonist, GW1229 (also known as 1229U91 or GR231118), is a potent Y_4 receptor agonist, and until now only a non-peptide antagonist, UR-AK49, was described for these receptors (Ziemek *et al.*, 2007).

The peripheral Y_4 receptor mRNA is highly expressed in the colon, small intestine and prostate (Lundell *et al.*, 1995). Furthermore, this receptor was detected within the brain but in lower levels (Lundell *et al.*, 1995; Parker & Herzog, 1999). Thus, in rodent brain, Y_4 receptor expression was identified in paraventricular hypothalamus nucleus, *nucleus tractus solitarius*, medial preoptic area and *area prostema* (Dumont *et al.*, 2004). Besides, the Y_4-ir present in the rat hypothalamus and cortex co-localizes with orexin-positive cell bodies and fibers, respectively (Campbell *et al.*, 2003). Moreover, in human brain, low levels of Y_4 receptor mRNA were found in the hypothalamus, hippocampus, subthalamic nucleus, thalamus and *substantia nigra*, *corpus callosum* and *caudate nucleus* (Yan *et al.*, 1996). It is interesting that certain areas like the hippocampus and amygdala exhibit the Y_4 receptor mRNA, but no protein has been reported (Parker & Herzog, 1999).

The activation of Y_4 receptor has major influence in the PNS, particularly in the gastrointestinal and reproductive system (Raposinho *et al.*, 2000; Moriya *et al.*, 2010). Furthermore, these receptors are also involved in the control of food intake (Sainsbury *et al.*, 2010).

1.3.4.5. Y_5 receptor

The sequence of human and rat Y_5 receptor is highly conserved between species but display only a 30-35% identity to the other NPY receptors (Gerald *et al.*, 1996; Hu *et al.*, 1996). Furthermore, the first cDNA cloned was called "Y_1-like receptor" because the corresponding gene appears as a residue on human chromosome 4q that is the same localization of the human Y_1 receptor, but in an opposite orientation (Gerald *et al.*, 1996; Hu *et al.*, 1996). Since mRNA for this receptor exist in several brain areas, including the hypothalamus, it becomes known as Y_5 or "feeding receptor" (Gerald *et al.*, 1996). The Y_5 receptor subtype is activated preferentially by NPY and PYY. Surprisingly, rat PP has very low affinity for the rat and human Y_5 receptor; however, human and bovine PP has similar affinity as NPY and PYY (Gerald *et al.*, 1996). To date, different selective Y_5 receptor agonists and antagonists have been reported. Indeed, the first NPY analog described as a selective Y_5 receptor agonist was [D-Trp^{32}]NPY, but soon realized that a specific agonist for this receptor subtype needed to contain a Ala^{31}-Aib^{32} motif. So, NPY analogs and PP/NPY chimera were synthesized with this motif and the [Ala^{31}-Aib^{32}]NPY (Cabrele *et al.*, 2000) is the most common. Concerning antagonists, CGP71683A compound was reported as the first selective antagonist of Y_5 receptor (King *et al.*, 1999), but then a more potent and selective inhibitor was synthesized, L-152,804 (Kanatani *et al.*, 2000).

At the peripheral level, Y_5 receptor is present in the intestine, ovary, testis, prostate, spleen, pancreas, kidney, skeletal muscle, liver, placenta and heart (Statnick *et al.*, 1998). Concerning the brain, it was demonstrated that, in rodents and humans, the Y_5 receptor mRNA is found in several brain regions, such as amygdala, *substantia nigra*, hypothalamus, cerebral cortex and hippocampus (Jacques *et al.*, 1996; Nichol *et al.*, 1999). Moreover, in the rodent and human hippocampal formation, these receptors have a stronger expression in DG sub-region than in pyramidal cell layers, CA3 and CA1 (Nichol *et al.*, 1999; Durkin *et al.*, 2000; Trivedi *et al.*, 2001). However, in the brain, the Y_5 receptor is probably less abundant than either the Y_1 or Y_2 receptors (Nichol *et al.*, 1999;

Durkin *et al.*, 2000). More recently, immunohistochemical analysis in rat brain have revealed that Y_1- and Y_5-ir co-localized in several brain regions, including cerebral cortex and hippocampus, and the presence of Y_5 in fibers suggests that activation of these receptors could modulate presynaptic neurotransmitter release (Wolak *et al.*, 2003).

There are many functions associated with Y_5 receptor activation, being the appetite regulation one of the most important (Cabrele *et al.*, 2000; Della-Zuana *et al.*, 2004). Also, this NPY receptor subtype has an important role as a modulator of the reproductive axis, circadian rhythmus and brain excitability (Woldbye *et al.*, 1997; Raposinho *et al.*, 1999; Gamble *et al.*, 2005).

1.3.4.6. y_6 receptor

The y_6 receptor gene was first cloned from mouse and rabbit libraries and its expression was found in brain areas, including hypothalamus and hippocampus (Gregor *et al.*, 1996; Matsumoto *et al.*, 1996). Despite the fact that this receptor is expressed in primate tissues, such as heart and skeletal muscles, it is a truncated non-functional protein (Gregor *et al.*, 1996; Matsumoto *et al.*, 1996). This suggests that, y_6 gene become nonfunctional during primate evolution.

The y_6 receptor shares the highest identity with the Y_1 subtype, but pharmacologically it is more close to the Y_2 receptor since it has high affinity for NPY(13-36) (Michel *et al.*, 1998). A physiological role for the cloned y_6 receptor has not yet been described, and so, the International Union of Pharmacology recommends that the y_6 receptor should be written by a lower-case “y” (Michel *et al.*, 1998).

A summary of NPY receptors characteristics is described in Table 1.2.

Table 1.2. *Characterization of NPY receptors (Silva, 2002)*

Receptor	Selective agonists	Antagonists	Signal transduction	Localization	Physiological role
Y_1	[Leu^{31},Pro^{34}]NPY; [Pro^{34}]NPY [Leu^{31},Pro^{34}]PYY; [Pro^{34}]PYY	BIBP3226; BIBO3304; SR120819A; GW1229; J-104870; PD160170; LY357897; H 409/22	$G_{i/o}$, Ca^{2+}, inhibition of adenylyl cyclase	Cortex, thalamus, hypothalamus, hippocampus, amygdala, heart, kidneys, intestines	Vasocontriction, anxiolysis, appetite regulation, ethanol consumption, locomotor activity
Y_2	NPY(13-36); NPY(3-36) PYY(13-36); PYY(3-36)	T_4-[NPY(33-36)]$_4$; BIIE0246; JNJ-31020028	$G_{i/o}$, Ca^{2+}, inhibition of adenylyl cyclase	Hippocampus, hypothalamus, amygdala, intestines, blood vessels	Memory process, neurotransmitter release, anticonvulsant, locomotor activity
Y_3	-	-	Ca^{2+}, inhibition of adenylyl cyclase	CA3, brainstem, NST, cardiac ventricular membranes, colon	Bradycardia
Y_4	PP	UR-AK49	G_i, Ca^{2+}, inhibition of adenylyl cyclase	Brainstem, hypothalamus, hipoccampus, colon, pancreas, testis, lung, intestines	Regulates pancreatic, gastric and small intestine secretions
Y_5	NPY(19-23)-(Gly^1,Ser^2,Gln^4,Thr^6, Ala^{31},Aib^{32},Gln^{34})-PP; [D-Trp^{32}]NPY	CGP71683A; L-152,804	G_i, Ca^{2+}, inhibition of adenylyl cyclase	Hippocampus, hypothalamus, cortex, thalamus, ovary, intestines, liver, heart, placenta, spleen, liver	Appetite regulation, anticonvulsant
y_6	-	-	Inhibition of adenylyl cyclase	Hippocampus, hypothalamus, heart, skeletal muscle	?

1.3.5. NPY-mediated signaling pathway

It has been shown that NPY receptors, namely Y_1, Y_2, Y_4 and Y_5, are coupled to inhibitory G-proteins (Gi) and thus mediate the inhibition of cyclic adenosine monophosphate (cAMP; Michel, 1991; Figure 1.17). Moreover, the activation of the G-protein complex by NPY can also lead to depressed Ca^{2+} channel and enhanced G-protein coupled inwardly rectifying potassium channel (GIRK) currents (Acuna-Goycolea *et al.*, 2005). So, while α subunit of Gi-protein inactivates adenylyl cyclase, the β/γ subunit activates a number of different kinase cascades. In fact, Y_1, Y_2, Y_4 and Y_5 receptors can also activate phospholipase C (PLC)- and phosphatidylinositol 3-kinase (PI3K)-related signaling pathways, such as MAPK/ERK cascade (Barnea & Roberts, 2001; Mullins *et al.*, 2002; Howell *et al.*, 2005). Hence, the activation of these cellular signaling cascades has a multitude of effects such as the transcription initiation and the stimulation or inhibition of hormone/neurotransmitter release.

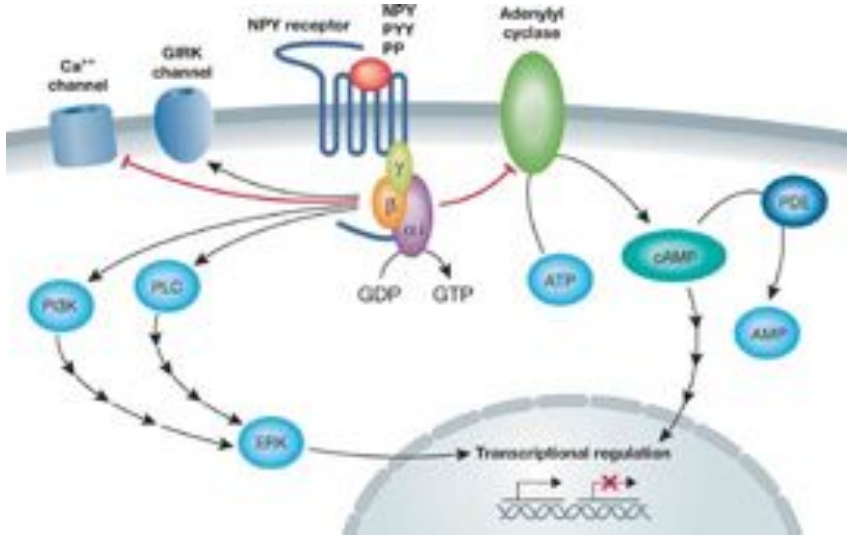


Figure 1.17. *Summary of the intracellular signaling cascades triggered by NPY receptors activation. Main abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; GDP, guanosine diphosphate; GIRK, G-protein coupled inwardly rectifying potassium channel; GTP, guanosine triphosphate; NPY, neuropeptide Y; PDE, phosphodiesterase; PI3K, phosphoinositide*

3-kinase; PLC, phospholipase C; PP, pancreatic peptide; PYY, peptide YY (Brothers & Wahlestedt, 2010).

1.3.6. NPY and brain disorders – focus on neuropathology of drugs of abuse

Many authors have demonstrated that NPY is involved in several neuropathologies, such as anxiety, depression, schizophrenia, epilepsy and Huntington's, Alzheimer's and Parkinson's diseases (Table 1.3). Moreover, pieces of evidence suggest that NPY has a neuroprotective role in brain disorders (Silva *et al.*, 2005a; Xapelli *et al.*, 2006; Decressac *et al.*, 2011). In fact, it was demonstrated that NPY is able to regulate excitotoxicity in the hippocampus, which is characterized by a supraphysiological stimulation of glutamate receptors followed by an increase of intracellular Ca^{2+} concentration that can lead to cell death. These events may be common features of a variety of neurological disorders (Plata-Salaman *et al.*, 2000; Silva *et al.*, 2003b). Moreover, our group has previously demonstrated that hippocampal neuronal death mediated by AMPA or KA was prevented by the activation of Y_1, Y_2 and Y_5 receptors (Silva *et al.*, 2003b). Importantly, the neuroprotective role of NPY seems to be due to its capacity to modulate the release of different neurotransmitters such as glutamate, GABA, DA, 5-HT (Silva *et al.*, 2005a).

Table 1.3. *NPYergic system changes in CNS disorders*

Disorder	NPYergic system changes
Epilepsy	• ⇑ NPY mRNA and protein levels in the rodent hippocampus, amygdala, striatum, enthorhinal cortex and extralimbic areas (Burazin & Gundlach, 1996; Sperk *et al.*, 1996; Nagaki *et al.*, 2000) • ⇑ Y_1, Y_2, and Y_5 mRNA level in mice DG (Christensen *et al.*, 2006) • ⇓ Y_1, Y_2 mRNA level in mice CA3 and CA1 (Christensen *et al.*, 2006) • ⇑ NPY-ir fibers in human hippocampus (Furtinger *et al.*, 2001) • ⇓ Y_1 and ⇑ Y_2 binding in human dentate hilus and molecular layer, respectively, (Furtinger *et al.*, 2001)
Alzheimer's disease	• ⇑ NPY-ir in rodent hippocampus and cortex (Diez *et al.*, 2003) • ⇓ NPY-ir and binding in human frontal and temporal cortices and hippocampus (Davies *et al.*, 1990; Martel *et al.*, 1990)
Huntington's disease	• ⇑ NPY-ir in human striatum and cortical regions (Beal *et al.*, 1988; Mazurek *et al.*, 1997)
Parkinson's disease	• ⇑ NPY mRNA expression in human striatum (Cannizzaro *et al.*, 2003) • ⇓ NPY levels in human cerebrospinal fluid (CSF) (Fall *et al.*, 1995)
Anxiety	• ⇑ NPY levels in rat striatum, hippocampus, hypothalamus and amygdala (Miller *et al.*, 2002; Primeaux *et al.*, 2005; Thorsell *et al.*, 2006) • ⇑ NPY levels in human plasma (Boulenger *et al.*, 1996)
Depression	• ⇓ NPY mRNA in rat nucleus accumbens and hippocampal CA sub-fields; ⇑ NPY mRNA in rat anterior nucleus and anterior cingulate cortex (Caberlotto *et al.*, 1998a) • ⇓ Y_1 mRNA in rat cortical regions and hippocampal dentate granular cells (Caberlotto *et al.*, 1998a) • ⇓ NPY levels in human CSF (Crespi, 2011) • ⇑ "low-expression NPY" genotype (Mickey *et al.*, 2011)
Schizophrenia	• ⇓ NPY mRNA in human cortical regions (Fung *et al.*, 2010) • ⇓ NPY-ir in human CSF (Peters *et al.*, 1990) • Morphological changes in NPY-positive fibers in human hippocampal CA4 sub-region (Iritani *et al.*, 2000)

Nowadays, drug abuse represents a significant health problem since originates serious neurologic-psychiatric complications, including depression, memory loss and cognitive decline. Furthermore, like the other neuropathologies abovementioned, it was reported that chronic consumption of drugs could induce alterations in NPYergic system (Westwood & Hanson, 1999; Thorsell, 2008). In addition, one study with human subjects observed that sequence variations in NPY receptors genes, especially Y_1, Y_2 and Y_5, are associated with alcohol and cocaine dependence (Wetherill *et al.*, 2008).

Ethanol is the most studied drug concerning NPYergic system. Actually, it was found that intragastric ethanol repeated treatment (4 days) leads to complex plastic changes in NPY and their receptor levels and activity in rat brain (Olling *et al.*, 2009). These authors demonstrated that during ethanol intoxication the mRNA levels of NPY was up-regulated in the hippocampus, but without differences in NPY receptors. During the withdrawal period the NPY levels were still increased in the hippocampus and there was an enhancement in piriform cortex (PirCx) and neocortex (NeoCx), as well as for the Y_1, Y_2 and Y_5 receptors mRNA levels in hippocampal and cortical regions (Olling *et al.*, 2009). Moreover, these alterations in NPY system are accompanied by an increase in total NPY receptors activity in both ethanol intoxication and withdrawal (Olling *et al.*, 2009). Besides, 8 weeks of chronic exposure to ethanol produced a significant effect on NPY-ir expression in amydgala and frontal cortex (Criado *et al.*, 2011). More recently, it was described that NPY signaling in the *nucleus accumbens core* modulates ethanol-induced behavioral sensitization since mice $NPY^{-/-}$ and Y_2 $receptor^{-/-}$ failed to display ethanol-induced behavioral sensitization (Hayes *et al.*, 2011). However, the treatment with NPY or Y_1 receptor agonist prevented the development of ethanol tolerance, as well as the withdrawal hyper-excitability (Bhisikar *et al.*, 2009).

Little is known about the role of NPYergic system under conditions of amphetamine-type stimulants consumption, namely amphetamine (AMPH), 3,4-Methylenedioxymethamphetamine (MDMA) and METH. Nevertheless, there are some studies showing that these psychostimulants altered NPY levels.

In fact, NPY expression seems to be susceptible to AMPH, since prolonged AMPH administration (10 mg/kg, twice daily, during 6 d) induced an up-regulation of NPY-ir in medial preoptic nucleus, pineal gland and plasma, and a reduction in *caudate putamen* and hypothalamus (Tessel *et al.*, 1985). More recently, it was observed that AMPH repeated treatment (5 mg/kg, twice daily for 6 d and once on day 7; s.c.) caused late (96h after the final dose) markedly and reversible decrease in NPY-ir in rat striatum, but not in the hippocampus (Obuchowicz *et al.*, 2005). In contrast, a single dose of AMPH (5 mg/kg; s.c.) had no effect in brain NPY levels (Obuchowicz *et al.*, 2005). In addition, upon a low single dose of AMPH (1.5 mg/kg), the Y_1 receptor antagonist modulated behavioural response to AMPH, e.g. inhibited the hyperactivity (Kask & Harro, 2000). Moreover, other work revealed that MDMA-induced cell death in mixed cultured of rat retinal neural cells was inhibited by NPY (Álvaro *et al.*, 2008). Interestingly, rat serum NPY levels showed a steady decrease in both treatment of MDMA and METH at different time points and dosages (Kobeissy *et al.*, 2008). Although only a few authors have been beholding the influence of METH in NPY, they all describe that this drug of abuse has a significant impact in the NPYergic system of the CNS. Thus, in rat paraventricular nucleus, it was described that NPY levels increased significantly just after 30-60 min and reached the maximum in 90-120 min following a single dose of METH (5 mg/kg; i.p.) (Yoshihara *et al.*, 1996). Recently, (Horner *et al.*, 2006) showed that both multiple doses (5 x 10 mg/kg at 6h intervals; i.p.) and a single high dose (15 mg/kg; i.p.) of METH increased the number of striatal neurons expressing pre-pro-NPY mRNA. Besides, the authors proposed that the increase of pre-pro-NPY mRNA-containing neurons is due to a mechanism mediated by DA D1 receptor, because the pretreatment with an antagonist of these receptors abolished the observed alterations in striatal neurons (Horner *et al.*, 2006). Other important study explored the neuroprotective role of NPY against METH-induced neurotoxicity in mice striatum (Thiriet *et al.*, 2005). So, in agreement with the previous studies, METH repeated treatment (4 x 10 mg/kg, 2-h intervals; i.p.) up-regulated the levels of NPY, and for the first time, this work showed that

also occurs a decrease and a biphasic alteration in Y_1 and Y_2 mRNA levels (Thiriet *et al.*, 2005). The same authors also found that NPY (10 μg; i.c.v.) protected against METH-induced neurotoxicity in mice striatum mainly through Y_2 receptors activation. These data were confirmed by studies showing that the toxic effects of METH were potentiated in the NPY KO mice (Thiriet *et al.*, 2005).

1.3.7. NPY in memory and learning

The NPY-like immunoreactivity, as described in section 1.3.3.2, has been shown to be present throughout the brain, but is concentrated in two principal structures: hypothalamus and hippocampus (Allen *et al.*, 1983; Chan-Palay *et al.*, 1986). Since the hippocampal formation has been repeatedly implicated in the modulation of cognition, this section will focus the putative role of NPY and their receptors in the regulation of cognitive function associated with learning and memory.

Pharmacological and electrophysiological studies have demonstrated that NPY modulates excitatory synaptic neurotransmission in the hippocampus (Malva *et al.*, 2003; Silva *et al.*, 2005a). In fact, through Y_2 receptors, NPY was able to inhibit glutamate release in the rat hippocampus (Silva *et al.*, 2005b). Besides, this effect seems to be associated with the reduction of Ca^{2+} influx into presynaptic nerve terminals across several types of voltage-gated Ca^{2+} channels, namely L-, N-, and P-/Q-types (Silva *et al.*, 2003a). Furthermore, the role of NPY on excitatory synaptic neurotransmission was suggested as responsible, at least in part, for its well-documented antiepileptic and antiepileptogenic properties (Xapelli *et al.*, 2006). However, increased levels of NPY, which has been linked with its anti-excitatory role, can affect other brain functions, such as learning and memory.

The precise involvement of NPY in cognitive processes is not yet clear, because the effect of this neuropeptide is dependent on the brain region (Flood *et al.*, 1989; Table 1.4). Indeed, in the dorsal hippocampus the overexpression of NPY increased the memory retention, while in the ventral hippocampus induced amnesia (Flood *et al.*, 1989). Moreover, there are studies reporting

that NPY may both improve and impair short-term working memory under delayed matching-to-sample and conditional discrimination (Thomas & Ahlers, 1991; Cleary *et al.*, 1994).

Table 1.4. *Comparative effect of NPY and NPY antibodies on learning and memory (Redrobe et al., 1999)*

Injection site	NPY	NPY antibodies
Dorsal hippocampus	Retention	Amnesic
Ventral hippocampus	Amnesic	Retention
Amygdala	Amnesic	Retention
Septal area	Retention	Amnesic

These effects are dependent on the concentration of NPY since low and high doses caused facilitation and impairment of memory processes, respectively (Thomas & Ahlers, 1991; Cleary *et al.*, 1994). Another work demonstrated that in rats, NPY injection (10 ng/ml, 5 μl; i.c.v.) significantly inhibited the induction and maintenance of long-term potentiation (LTP) in granule cell synapses resulting in memory deficits (Whittaker *et al.*, 1999). In addition, the same authors observed that the effect of NPY on LTP was coupled with the reduction of glutamate release and JNK protein levels up-regulation in hippocampal synaptosomes. However, the overexpression of NPY also affects the high-frequency activation of CA1-subicular synapses. Indeed, in NPY transgenic mice there was an attenuation of excitatory synaptic transmission in CA1 sub-region (Sorensen *et al.*, 2008b). Later, the same group reported that hippocampal NPY gene transfer into the intact brain slightly altered short-term synaptic plasticity affecting the LTP in the hippocampal CA1 sub-region and, consequently, the rate of hippocampal–based spatial discrimination learning (Sorensen *et al.*, 2008a). Furthermore, they also observed that the viral-mediated long-lasting overexpression of NPY in the hippocampus decreases Y_1 receptor binding, while Y_2 receptor binding is unaltered (Sorensen *et al.*, 2008a). In agreement, transgenic rats overexpressing NPY showed increase levels of pre-pro-NPY mRNA and NPY peptide in hippocampus, accompanied by decreased Y_1 receptor

activity, but no alteration in Y_2 receptor signaling has been observed (Thorsell *et al.*, 2000). Previously, several studies have been demonstrated that NPY within the hippocampal formation acts mainly at presynaptic Y_2 sites to reduce presynaptic Ca^{2+} entry, inhibit glutamatergic transmission and so suppress the formation of LTP (Whittaker *et al.*, 1999; Silva *et al.*, 2003a, 2005b). Hence, taken together, regarding the studies abovementioned, it is plausible to attribute the inhibition of excitatory synaptic activity to Y_2-mediated signaling. Accordingly, application of the Y_2 receptor antagonist, BIIE0246, prevented the inhibition of recurrent mossy fibers synaptic transmission mediated by spontaneous release of NPY in both mice and rats (Tu *et al.*, 2005, 2006).

On other hand, in some brain regions, such as dorsal hippocampal and septal area (Table 1.4), NPY enhanced the memory retention (Flood *et al.*, 1989). Interestingly, mice-treated with Y_1 receptor agonist, [Leu^{31},Pro^{34}]NPY, showed attenuation of learning impairments induced by noncompetitive NMDA receptor channel antagonist, MK-801 (Bouchard *et al.*, 1997). Furthermore, Redrobe *et al.* (2004) characterized Y_2 receptor KO mice regarding learning and memory processing and found that these animals exhibited deficits in the Morris water maze and object recognition task.

Despite previous studies, the precise role of NPY and, in particular, the role of the Y_2 receptor subtype in learning and memory processing remains to be fully characterized.

1.4. Objectives

Despite the extensive evidence that METH is neurotoxic, the mechanisms underlying those effects are still not well understood. The most studied hypotheses focused on intra-neuronal events, such as DA oxidation, oxidative stress and excitotoxicity in the striatum (Cadet & Krasnova, 2009; Silva *et al.*, 2010). However, recent reports suggested that glial cells may also contribute to METH-induced neuropathology (Yamaguchi *et al.*, 1991; LaVoie *et al.*, 2004;

Nakajima *et al.*, 2004; Sriram *et al.*, 2006a; Sriram *et al.*, 2006b; Simões *et al.*, 2007; Cadet & Krasnova, 2009), and that hippocampus is highly susceptible to this drug of abuse (Thompson *et al.*, 2004; Swant *et al.*, 2010; Kim *et al.*, 2011a; Martins *et al.*, 2011). Nevertheless, many questions remain to be answered.

So, we first investigated whether an acute administration of METH induces an inflammatory response by analyzing the mRNA alterations of the pro-inflammatory cytokines IL-1β, IL-6, and TNF- α in the mice hippocampus, frontal cortex, and striatum (Chapter 2). Then, we explored the effect of the same METH treatment on mice hippocampal glial cells and neurons. Since microglia and astrocytes are the main source of pro-inflammatory cytokines, it was also evaluated the possible alteration in protein levels of TNF-α and their receptors, TNFR1 and TNFR2, mediated by METH administration. Moreover, a new protective strategy was used against METH-induced hippocampal neuropathology, consisting in the administration of an anti-inflammatory drug, indomethacin (Chapter 3). Nonetheless, neuroinflammation seems to have opposite effects, contributing to CNS injury or being involved in some mechanisms of brain repair (Farooqui *et al.*, 2007; Belanger & Magistretti, 2009; Czeh *et al.*, 2011). In particular, it has been reported that TNF-α displays a dual role under conditions of hippocampal neurotoxicity (Bernardino *et al.*, 2005b). Thus, in Chapter 4, we explored the role of TNF-α and its receptors on METH-induced hippocampal neurodegeneration. Since microglia is an important immune effector in CNS, we also clarified the direct effect of METH on microglial cells and its impact in the endogenous TNF system. Besides, we aimed to evaluate the role of exogenous TNF in the microglial toxicity induced by METH.

Recently, a new target has been raised as a neuroprotector factor against METH-induced striatal cell death: the neuropeptide Y (Thiriet *et al.*, 2005). In fact, this peptide has been previously identified as an important neuroprotective agent in the hippocampus under excitotoxic conditions (Silva *et al.*, 2003b; Xapelli *et al.*, 2007). However, the impact of METH on the hippocampal NPY system and its consequences remain unknown. So, in Chapter

5, our goal was to clarify if METH intoxication affects the NPY system in the mouse hippocampus. In addition, we explored how it may compromise memory processes. Finally, we aimed to understand if NPY is neuroprotective against METH-induced hippocampal cell death and microglial activation (Chapter 6).

Overall, our main aim was to better understand the impact of METH in brain function, focusing on neuroinflammation and NPYergic system alterations. In the end, our goal is to provide a therapeutic target to prevent, or at least to diminish, the neurotoxic effects induced by METH.

Chapter 2

Methamphetamine-induced early increase of IL-6 and TNF-α mRNA expression in the mouse brain

2.1. Abstract

The mechanisms by which methamphetamine (METH) cause neurotoxicity are not well understood. Recent studies have suggested that METH-induced neuropathology may result from a multicellular response in which glial cells play a prominent role, and so is plausible to suggest that cytokines may participate in the toxic effects of METH. Therefore, in the present work we evaluated the effect of an acute administration of METH (30 mg/kg; a single intraperitoneal injection) on the interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α mRNA expression levels in the mice hippocampus, frontal cortex and striatum. We observed that METH did not induce changes in the IL-1β mRNA expression levels in both hippocampus and striatum, with immeasurable levels in the frontal cortex. Regarding IL-6, METH induced an increase in the expression levels of this cytokine in the hippocampus and striatum, 1h and 30 min post-injection, respectively. In the frontal cortex, the increase in IL-6 mRNA levels was more significant and remained high even after 2h. Moreover, the expression levels of TNF-α were increased in both hippocampus and frontal cortex 30 min post-METH administration, with immeasurable levels in the striatum. We conclude that the pro-inflammatory cytokines IL-6 and TNF-α rapidly increase following METH administration, providing a new insight for understanding the effect of this drug of abuse in the brain.

2.2. Introduction

Methamphetamine (METH) is an addictive stimulant drug that steadily gained popularity and its consumption can cause serious psychiatric and neurological symptoms (Lan *et al.*, 1998). Studies with human METH abusers have reported a selective pattern of cerebral deterioration that contributes to impaired memory performance (Thompson *et al.*, 2004). Moreover, acute METH intoxication can result in stroke, cardiac arrhythmia, malignant hyperthermia, cardiovascular collapse, and because of the long duration of action of METH, these acute medical complications can present serious management challenges.

It is believed that the pathogenesis of METH-induced neurotoxicity is in part identical with other neurodegenerative disorders such as Alzheimer´s and Parkinson´s disease. Although a number of factors appear to contribute to METH-induced neurotoxicity, the exact mechanisms are still unknown. Many of the hypotheses have focused on intra-neuronal events such as dopamine oxidation (Pereira *et al.*, 2002, 2006), oxidative stress, and excitotoxicity (Cadet *et al.*, 2007). Indeed, METH noxious effects on the brain have long been associated to dopamine (DA) and serotonin (5-HT) (Yamamoto & Bankson, 2005), but over the last decades the focus has been shifting towards glutamate (Glu). Moreover, we have very recently demonstrated that METH induces significant alterations on hippocampal n-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptor subunit levels (Simões *et al.*, 2007). Despite these evidences, recent studies have suggested that METH-induced neuropathology may also result from a multicellular response in which glial cells play a prominent role (LaVoie *et al.*, 2004; Bowyer & Ali, 2006; Kuhn *et al.*, 2006; Sriram *et al.*, 2006b). Several observations suggest that conversion of glial cells to their “reactive” state, and the associated increase in expression of cytokines and chemokines, may play a role in neurodegeneration. Thus, is plausible to

suggest that other molecules, such as cytokines, might also participate in the toxic effects of METH.

Cytokines, a diverse group of polypeptides that are generally associated with inflammation, immune activation, and cell differentiation or death, have diverse actions, and most have little or no known function in healthy tissues, being rapidly induced in response to tissue injury or inflammation (Allan & Rothwell, 2001). Indeed, cytokines have beneficial or detrimental effects on cell survival, as we have previously observed that interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) act as biphasic modulators of AMPA-induced neurodegeneration (Bernardino *et al.*, 2005b). Thus, changes in cytokine levels may reflect an attempt to counteract the degeneration by initiating protective and/or regenerative mechanisms. It is known that IL-1β is among cytokines the first to be synthesized by microglia, in moderate levels, in response to an insult, which in turn stimulates the proliferation of astrocytes and the consequent release of interleukin-6 (IL-6; Norris *et al.*, 1994; Ritchie *et al.*, 1996) that is capable to modulate the differentiation and survival of neurons. Similarly, IL-1β can induce TNF-α production that in turn acts synergistically with IL-1β to induce IL-6 expression (Benveniste *et al.*, 1990; Bethea *et al.*, 1992), and then IL-6 inhibits the synthesis of TNF-α.

Recently, it was shown that IL-6 null genotype affords protection to DA and 5-HT terminals damage, apoptotic cell death and reactive gliosis induced by METH (Ladenheim *et al.*, 2000). Other authors have shown an increase of IL-1β mRNA in METH-treated animals, which may be partly involved in the activation of IL-6 cascade and consequently astrogliosis (Yamaguchi *et al.*, 1991). Clearly, astrogliosis play a role in METH-induced hippocampal dysfunction (Simões *et al.*, 2007) and microglia is activated in response to METH administration (LaVoie *et al.*, 2004). However, attenuation of microglia activation is not by itself enough to afford neuroprotection (Sriram *et al.*, 2006b). Moreover, recent findings suggested that TNF-α plays a neuroprotective role in METH-induced drug dependence and neurotoxicity by activating plasmalemmal and vesicular

DA transporter, as well as by inhibiting METH-induced increase in extracellular DA levels (Nakajima *et al.*, 2004).

In the present study, we investigated if an acute administration of METH induces an inflammatory process by analyzing the mRNA alterations of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the mice hippocampus, frontal cortex and striatum.

2.3. Materials and Methods

2.3.1. Animals and drug treatment

Four-month-old male wild-type C57BL/6J mice (Charles River Laboratories, Inc, Spain, Barcelona) were housed under standard 12:12h light-dark with ad libitum access to food and water. Mice were administrated intraperitoneally (i.p.) with METH (30 mg/kg body weight) (Faculty of Pharmacy, University of Porto, Porto, Portugal) dissolved in a maximum volume of 100 µl of sterile 0.9% NaCl, and were sacrificed 30 min, 1h and 2h post-injection. The control group was administered with 100 µl of sterile 0.9% NaCl and sacrificed 2h post-injection. Moreover, to induce inflammation (positive control), mice were injected (i.p.) with 5 mg/kg LPS (Sigma, St Louis, MO, USA) and were sacrificed 4h post-administration.

Experimental procedures were performed accordingly to the guidelines of the European Community for the use of animals in the laboratory. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3.2. Isolation of total RNA

Total RNA was isolated from the mice hippocampus, frontal cortex and striatum using TRI REAGENT (Sigma-Aldrich, St Louis, MO, USA) according to manufacture's instructions. Briefly, tissue was homogenized in guanidium thiocyanate and phenol. Chloroform was added and after centrifuging (12500

rpm, 15 min, 4ºC), the RNA was isolated in the aqueous phase and precipitated with isopropanol. The pellet was then washed with 75% ethanol, dried and redissolved in diethylpyrocarbonate (DEPC)-treated water (Fluka, Sigma-Aldrich, Switzerland). The total amount of RNA was quantified by optical density (OD) measurements at 260 nm, and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm using RNA/DNA calculator GeneQuant II (Pharmacia Biotech Amersham Biosciences AB, Uppsala, Sweden).

2.3.3. RT-PCR analyses

Pro-inflammatory cytokines IL-1β, IL-6 and TNF-α mRNA levels were determined by reverse transcriptase-PCR (RT-PCR). cDNA was transcribed from 2 µg RNA of the hippocampus, frontal cortex and striatum, using Oligo dT(15) primers (Bioron, Ludwigshafen, Germany). Afterwards, PCR was performed in a 50 µl reaction volume containing 5 µl (IL-1β, IL-6 and β-actin) or 10 µl (TNF-α) cDNA, 1 µl dNTP mix, 5 µl 10x complete buffer for Taq DNA polymerase, 0.25 µl Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 0.2 µl of both forward and reverse primers, and a variable volume of water. The primers used in PCR reactions were described in Table 2.1 (all presented as 5'-3'; MWG-Biotech AG, Ebersberg, Germany).

Table 2.1. *List of primers used in RT-PCR*

Cytokine	Primers	
	Forward	**Reverse**
IL-1β	ATGGCAACTGTTCCTGAACTCAACT	AGGACAGGTATAGATTCTTTCCTT
IL-6	CAAGAGACTTCCATCCAGTTGCCT	TTTCTCATTTCCACGATTTCCCAG
TNF-α	CTGTAGCCCACGTCGTAGCA	CGGCAGAGAGGAGGTTGACT
β-actin	GACTACCTCATGAAGATCCT	ATCTTGATCATGGTGCTG

The PCR cycling profile for IL-1β andβ-actin was 1 min at 95ºC for denaturation, annealing at 58ºC for 1 min, extension at 72ºC for 1 min for 38 cycles and a 10 min final extension period at 72ºC. For IL-6 and TNF-α, the PCR

cycling profile was 1 min at 95ºC for denaturation, annealing at 60ºC (IL-6)/58ºC (TNF-α) for 30 sec, extension at 72ºC for 30 sec for 38 cycles and a 10 min final extension period at 72ºC. The mRNA levels of β-actin were used as a loading control, and negative controls were performed to ensure that PCR products result from RNA transcription (data not shown).

PCR products were separated by gel electrophoresis (1.5% agarose) and stained with ethidium bromide. Densitometrical analysis was performed on Universal Hood II (Bio-Rad Laboratories, Milan, Italy) and mRNA expression was evaluated by the band-intensity ration of control versus METH-injected mice using the ImageQuant 5.0 software.

2.3.4. Statistical analyses

The data are expressed as means ± SEM. Statistics were performed using one-way ANOVA, followed by Dunnett's post-test, as indicated in the figure legends. The significance level was set at $p < 0.05$.

2.4. Results

The acute administration of 30 mg/kg METH did not induce statistically significant changes in the IL-1β mRNA expression levels in both hippocampus and striatum, and neither at the different time points analyzed (Figure 2.1). In the hippocampus, IL-1β mRNA expression levels at 30 min, 1h and 2h post-injection were 129.1 ± 8.8%, 100.1 ± 4.5% and 109.2 ± 2.9% of control, respectively (Figure 2.1A). Also, the injection of 5 mg/kg LPS did not induce a significant change in the pro-inflammatory cytokine levels (103.2 ± 19.6% of control) (Figure 2.1A). Moreover, in the striatum (Figure 2.1B), IL-1β mRNA expression levels at 30 min, 1h and 2h post-injection were 79.6 ± 11.2%, 98.2 ± 7.6% and 69.7 ± 6.9%, respectively, and again, the administration of LPS resulted in IL-1 β mRNA expression levels similar to the control (106.7 ± 7.6% of control; Figure 2.1B). In contrast with the results obtained in both hippocampus

and striatum, the expression of IL-1β mRNA was not detected in the frontal cortex under the same experimental conditions (data not show).

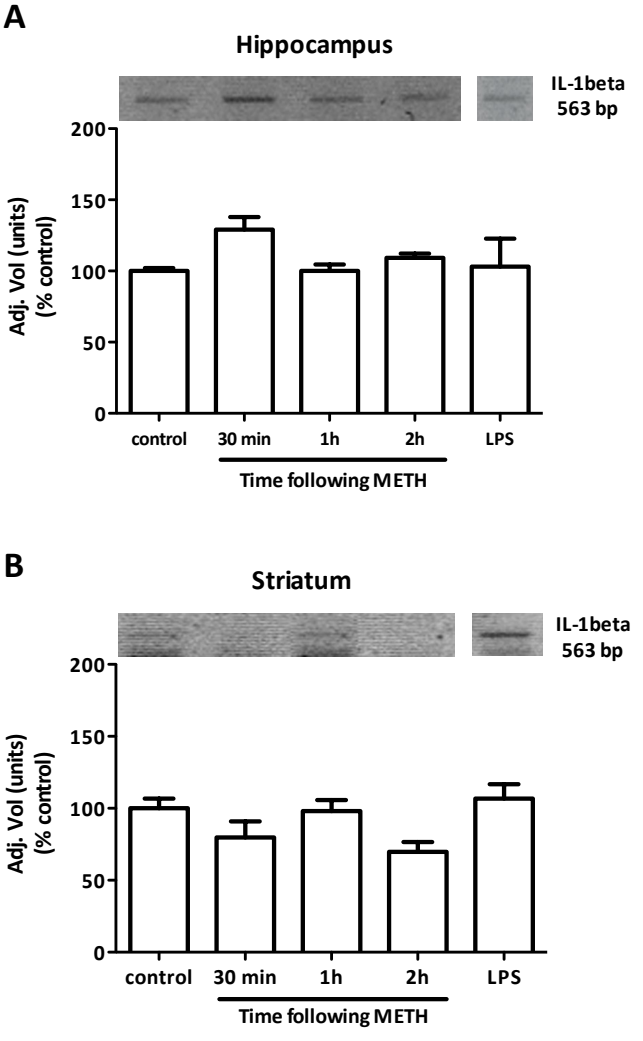
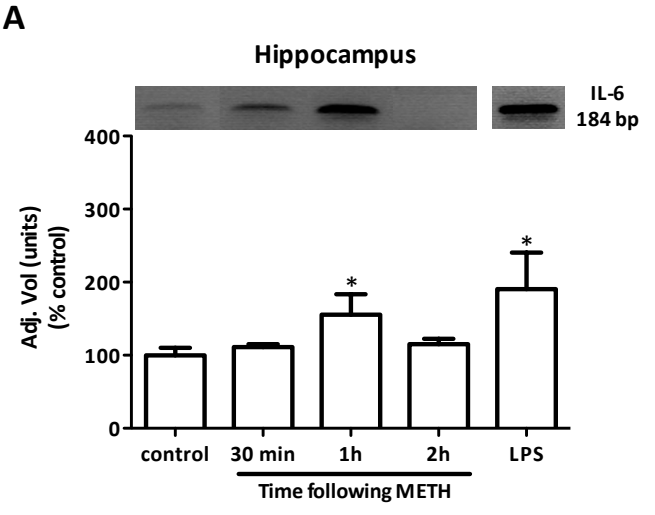


Figure 2.1. *Expression levels of IL-1β mRNA in mice hippocampus (A) and striatum (B) after acute administration of METH (30 mg/kg). The mRNA levels were analyzed 30 min, 1h and 2h post-administration, by RT-PCR. The control animals were given 0.9% NaCl solution and sacrificed 2h later. As a positive control we used the mRNA obtained from mice injected with 5 mg/kg LPS and sacrificed 4h later. The IL-1β mRNA expression was measured using ImageQuant 5.0 and each value represents the means ± SEM of three mice.*

Concerning IL-6, the changes induced by METH were highly significant in the three brain regions analyzed (Figure 2.2). In the hippocampus, IL-6 mRNA

values were very similar to the control at 30 min post-METH administration (111.2 ± 3.9% of control; Figure 2.2A). Only after 1h was possible to observe an increase of IL-6 mRNA expression levels to 155.5 ± 28.2% of control ($p < 0.05$) recovering to basal levels after 2h (115.1 ± 7.3% of control). Moreover, LPS induced a significant increase of IL-6 mRNA levels to 190.5 ± 49.9% of control (Figure 2.2A). Interestingly, in the frontal cortex the effect induced by METH was very significant at both 30 min and 1h post-injection (270.9 ± 18.8% and 304.8 ± 7.2% of control, respectively; $p < 0.01$), and the cytokine levels remained increased even after 2h (211.7 ± 42.1% of control; $p < 0.05$; Figure 2.2B). Also, 5 mg/kg LPS induced a very significant increase of IL-6 levels (338.0 ± 26.6%; $p < 0.001$; Figure 2.2B). In the striatum, we could observe a significant increase of IL-6 mRNA levels at 30 min and 1h after METH administration (129.9 ± 6.7% and 147.2 ± 0.1% of control, respectively; $p < 0.05$), whereas 2h post-METH or LPS administration, the values were not statistically different from control (129.9 ± 6.7%, 75.3 ± 11.5% or 139.7 ± 24.9% control, respectively; Figure 2.2C).



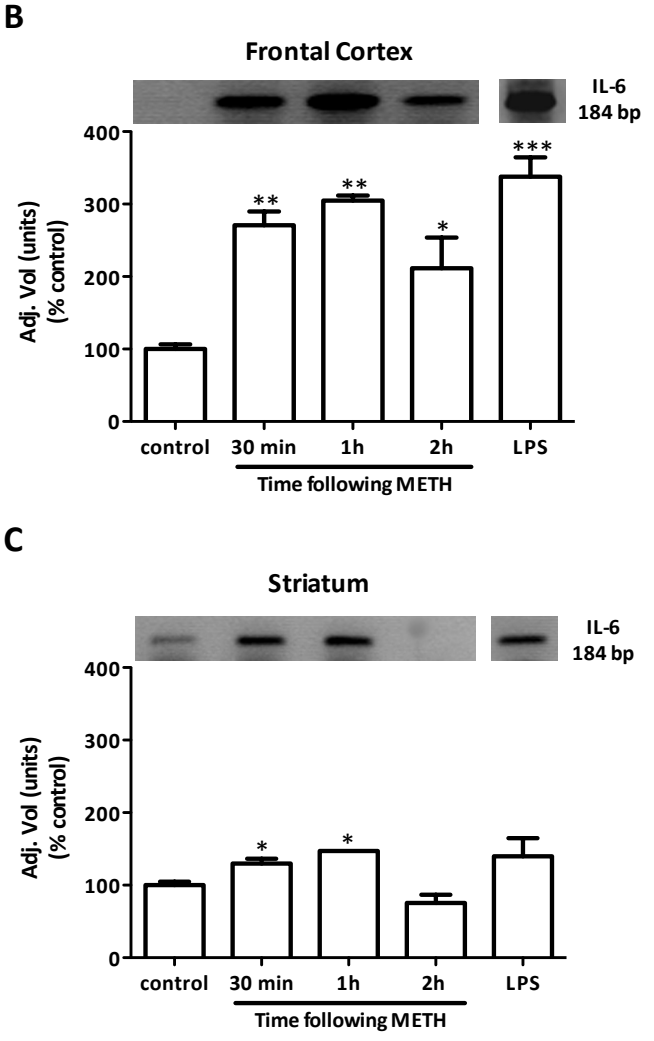


Figure 2.2. *Acute administration of METH (30 mg/kg) induces significant alterations of IL-6 mRNA expression levels in the hippocampus (A; see previous page), frontal cortex (B) and striatum (C). The mRNA levels were analyzed 30 min, 1h and 2h post-administration, by RT-PCR. The control animals were administered with 0.9% NaCl and sacrificed 2h later. As a positive control we used the mRNA obtained from mice injected with 5 mg/kg LPS and sacrificed 4h later. The IL-6 mRNA expression was quantified using ImageQuant 5.0 and each value represents the means ± SEM of four mice.* $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *- Dunnett´s post hoc; statistical significance when compared to control (NaCl administration).*

Despite the fact that no expression levels of TNF-α was detected in the striatum (data not shown), we could observe a significant increase of TNF-α mRNA expression levels in the hippocampus 30 min post-METH administration (151.3 ± 8.6% of control; $p < 0.05$; Figure 2.3A), and in the frontal cortex 30 min

and 1h after METH administration (147.0 ± 6.0% and 174.3 ± 26.6% of control, respectively; $p < 0.05$; Figure 2.3B). Moreover, the administration of LPS also induced a significant increase of TNF-α levels in the hippocampus (172.0 ± 27.7% of control; $p < 0.05$; Figure 2.3A), but not in the frontal cortex (136.7 ± 11.0% of control; Figure 2.3B). At 1h and 2h post-METH administration the TNF- α levels in the hippocampus were 86.0 ± 1.1% and 89.8 ± 9.3% of control, respectively (Figure 2.3A), and in the frontal cortex, 30 min and 2h after METH administration, the levels of the pro-inflammatory cytokine were 147.0 ± 6.0% and 110.2 ± 0.7% of control, respectively (Figure 2.3B).

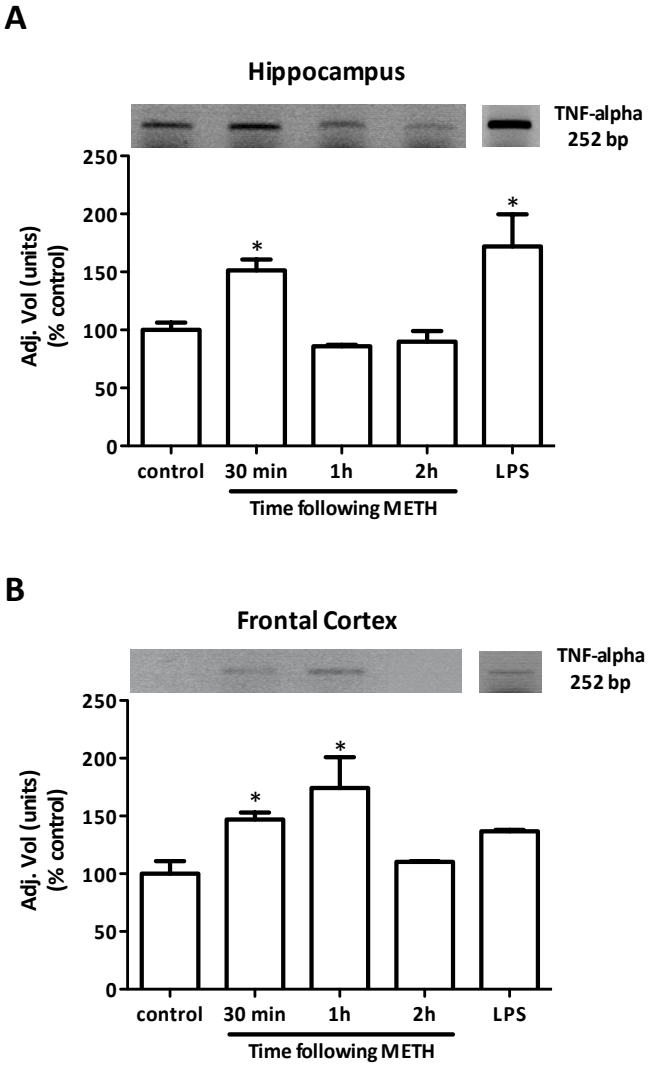


Figure 2.3. *Alterations of total mice hippocampal (A) and frontal cortex (B) TNF-α mRNA expression levels following METH (30 mg/kg) administration. The mRNA levels were examined 30 min, 1h and 2h post-injection, by RT-PCR. The control animals were administered with 0.9% NaCl solution and sacrificed 2h later. As a positive control, we used the mRNA obtained from mice injected with 5 mg/kg LPS and sacrificed 4h later. Quantification of mRNA levels was performed by ImageQuant 5.0 and each value represents the means ± SEM of at least three mice.* $^{*}p < 0.05$*, Dunnett´s post hoc; statistical significance when compared to control (NaCl administration).*

2.5. Discussion

It has been suggested that cytokines are involved in the effects of psychostimulants in the central nervous system. Indeed, a high single dose of METH induces TNF-α mRNA and protein expression in the mice brain (Flora *et al.*, 2002), whereas others showed that chronic low doses, but not acute treatment, increases TNF-α expression (Nakajima *et al.*, 2004). Moreover, the neurotoxicity induced by a repeated METH administration was attenuated in mice with a null mutation for IL-6 (Ladenheim *et al.*, 2000), and a single METH injection causes a marked induction of hypothalamic interleukin-1β mRNA in mice (Yamaguchi *et al.*, 1991; Halladay *et al.*, 2003; Numachi *et al.*, 2007). Here, we investigated the effect of an acute high dose of METH (30 mg/kg) on the pro-inflammatory cytokines levels in the hippocampus, frontal cortex and striatum. We showed that this drug of abuse does not induce changes in IL-1β mRNA levels in both hippocampus and striatum, being the mRNA expression levels very low under physiological conditions. Nonetheless, it was observed a small, but not statistically different, increase in hippocampal IL-1β mRNA levels at 30 min post-METH administration. Several studies have demonstrated that, when this cytokine is released in modest concentrations, it enhances the capacity to modulate the differentiation and survival of neuronal cells by activating the astrocytes (Parish *et al.*, 2002; Basu *et al.*, 2004; Liberto *et al.*, 2004). Indeed, some authors have shown that the production of IL-1β by microglial cells, after a brain insult, stimulates the proliferation of astrocytes and thereby the consequent release of IL-6 (Norris *et al.*, 1994; Ritchie *et al.*, 1996). Moreover, our group has previously observed that IL-1β and TNF-α act as biphasic modulators of AMPA-induced neurodegeneration since either potentiation of excitotoxicity or neuroprotection was observed, depending on the concentration of the cytokines and the timing of exposure (Bernardino *et al.*, 2005b). In the present study, we also demonstrated that administration of LPS did not induce an increase of IL-1β mRNA levels, at least between 1h and 4h

post-injection. In accordance with our results, Li and collaborators (Li *et al.*, 2007) showed that LPS-activated microglia result in significant upregulation of IL-1β expression only after 8h of exposure, and the level of expression was not so high as other pro-inflammatory cytokines, such as IL-6 and TNF-α.

The precise role of IL-6 in response to injury remains unclear. In some instances IL-6 appears to exert neuroprotective effects (Penkowa *et al.*, 2000; Penkowa & Hidalgo, 2000; Yamashita *et al.*, 2005) but in other studies it has been shown to promote degenerative mechanisms (Quintanilla *et al.*, 2004). Regarding this pro-inflammatory cytokine, we observed that mRNA expression levels were very rapidly increased in all brain regions analyzed, being, however, more prominent in the frontal cortex. Thus, the augment of IL-6 by administration of METH can lead us to different interpretations. Ladenheim and collaborators (2004) demonstrated that METH-induced damage of DA and 5-HT terminals, apoptotic cell death and reactive gliosis are attenuated in IL-6 knockout mice, suggesting that IL-6 might be an important component of the toxic cascade caused by METH. In addition, others have reported that IL-6 deficiency conferred protection from disruption in working memory induced by LPS (Sparkman *et al.*, 2006) and attenuates sensitivity to the depressing effects of LPS and IL-1 on social exploration and body weight (Bluthe *et al.*, 2000). Furthermore, circulating IL-6 is positively correlated with deficits in cognition in humans (Weaver *et al.*, 2002). Indeed, Balschun and collaborators (2004) suggested that IL-6 may sit in a pivotal position and serve as an important catalyst because administration of IL-6 neutralizing antibodies prolonged long-term potentiation (LTP) and improved spatial alternation behavior. Several studies have, indeed, demonstrated that cytokines have important normative functions in learning and memory and that perturbation of steady-state levels may alter cognitive processes. On the other hand, it was demonstrated that IL-6, as well as IL-1β, are required for a cytotrophic response to support sprouting of dopaminergic neurons, because glial response induced by these cytokines is essential in the regulation of axonal sprouting after injury (Liberto *et al.*, 2004). A more recent study also provides strong evidences that IL-6 promotes axonal

regrowth and network repair of CNS tissue following a lesion, using an in vitro model of lesion in hippocampal slice cultures (Hakkoum *et al.*, 2007). Regarding our results, they suggest an important role for IL-6 under conditions of METH consumption, since mRNA levels were significantly increased in the brain. However, the source and the consequence(s) of such increase remain unknown.

Brain TNF-α levels are typically increased in a variety of CNS disorders, including trauma (Goodman *et al.*, 1990), ischemia (Liu *et al.*, 1994), multiple sclerosis (Rieckmann *et al.*, 1994) and temporal lobe epilepsy (Vezzani *et al.*, 2002; Bernardino *et al.*, 2005a). Moreover, several studies demonstrated that treatment with METH increases TNF-α mRNA (Flora *et al.*, 2002; Nakajima *et al.*, 2004; Nomura *et al.*, 2006). Indeed, Nakajima and collaborators (Nakajima *et al.*, 2004) demonstrated that, although acute treatment at a dose of 2 mg/kg did not change TNF-α gene expression, a repeated treatment with METH (2 mg/kg for 5 days) induces a significant increase in TNF-α mRNA and protein expression in the rat brain. In contrast, we presently show that a single high dose of METH induces a significant increase in TNF-α mRNA levels in both hippocampus and frontal cortex, just after 30 min and 1h, respectively. Although TNF-α has been suggested to be toxic to neurons and glia, and to be correlated with neurotoxicity induced by METH (Nomura *et al.*, 2006), recent studies have also demonstrated neuroprotective effects of this cytokine. Indeed, the up-regulation of TNF-α has been associated with a neuroprotective role in METH-induce drug dependence and neurotoxicity by activating plasmalemmal and vesicular DA transporter as well as by increasing extracellular DA levels (Nakajima *et al.*, 2004). Moreover, the same authors clearly showed that exogenous TNF-α blocked locomotor-stimulating and rewarding effects of METH, as well as METH-induced dopaminergic neurotoxicity in mice following a chronic administration (4mg/kg; four times at 2h intervals) (Nakajima *et al.*, 2004). The dual role, i.e. toxic and protective, of TNF-α will be explored by us under conditions of acute METH administration.

In conclusion, we demonstrated that an acute high dose of METH induces an early increase in the expression levels of IL-6 mRNA in the hippocampus,

frontal cortex and striatum, and TNF-α mRNA only in the hippocampus and frontal cortex. Studies are under way in our laboratory to understand the beneficial/detrimental effects of IL-6 and TNF-α on cell survival under conditions of METH administration.

Chapter 3

Methamphetamine-induced neuroinflammation and neuronal dysfunction in the mice hippocampus: preventive effect of indomethacin

3.1. Abstract

Methamphetamine (METH) causes irreversible damage to brain cells leading to neurological and psychiatric abnormalities. However, the mechanisms underlying life-threatening effects of acute METH intoxication remain unclear. Indeed, most of the hypotheses focused on intra-neuronal events such as dopamine oxidation, oxidative stress and excitotoxicity. Yet, recent reports suggested that glia might contribute to METH-induced neuropathology. In the present study, we investigated the hippocampal dysfunction induced by an acute high dose of METH [30 mg/kg; intraperitoneal injection (i.p.)], focusing on the inflammatory process and changes in several neuronal structural proteins. For that, 3-month-old male wild-type C57BL/6J mice were killed at different time-points post-METH. We observed that METH caused an inflammatory response characterized by astrocytic and microglia reactivity and tumor necrosis factor (TNF) system alterations. Indeed, glial fibrillary acidic protein (GFAP) and CD11b immunoreactivity were up-regulated, likewise TNF-α and TNF receptor 1 protein levels. Furthermore, the effect of METH on hippocampal neurons was also investigated and we observed a down-regulation in beta III tubulin expression. To clarify the possible neuronal dysfunction induced by METH, several neuronal proteins were analyzed. Syntaxin-1, calbindin D28k and tau protein levels were down-regulated whereas synpatophysin was up-regulated. We also evaluated whether an anti-inflammatory drug could prevent or diminish METH-induced neuroinflammation, and we concluded that indomethacin (10 mg/kg; i.p.) prevented METH-induced glia activation and both TNF system and beta III tubulin alterations. In conclusion, we demonstrated that METH triggers an inflammatory process and leads to neuronal dysfunction in the hippocampus, which can be prevented by an anti-inflammatory treatment.

3.2. Introduction

Methamphetamine (METH) is a highly addictive psychostimulant, which consumption has substantially increased all over the world. This drug of abuse leads to dysfunction of several brain regions and most of the studies have been extensible exploring the toxic effects of METH in the striatum. However, there are only few and recent evidences that the hippocampus is highly susceptible to this drug. Thompson and collaborators showed that METH leads to memory deficit, which is correlated, with a decrease of hippocampal volume and white-matter hypertrophy. Moreover, METH-induced impaired memory performance was associated with decreases in dopamine transporter activity (McCann *et al.*, 2008). Several evidences have also suggested that METH-induce neurotoxicity involves the activation of calpains and caspases (Gold *et al.*, 2009; Krasnova & Cadet, 2009), production of reactive oxygen and nitrogen species with a subsequent induction of oxidative stress and the release of high levels of glutamate causing excitotoxicity (Quinton & Yamamoto, 2006; Cadet *et al.*, 2007; Krasnova & Cadet, 2009).

More recently, some authors have suggested that METH induces glial activation (LaVoie *et al.*, 2004; Simões *et al.*, 2007; Fantegrossi *et al.*, 2008), which also contributes to METH-related neuropathology. Indeed, it was demonstrated that a single high dose of METH increases tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) mRNA levels in mouse striatum (Sriram *et al.*, 2006b), and interleukin-1beta (IL-1-β) mRNA levels in the rat hypothalamus (Yamaguchi *et al.*, 1991). Accordingly, we also showed that an acute METH treatment (30 mg/kg, i.p.) induced an increase in TNF-α and IL-6 mRNA levels in mice hippocampus, without changes in the IL-1β mRNA levels (Gonçalves *et al.*, 2008). In fact, the pro-inflammatory cytokine TNF-α appears to exhibit a dual role in the brain (Bernardino *et al.*, 2005b; Sriram & O'Callaghan, 2007) but are typically increased in several central nervous system (CNS) disorders, such as epilepsy (Lehtimaki *et al.*, 2003) and ischemia (Tuttolomondo *et al.*, 2008).

Regarding to METH insult, TNF-α can have beneficial or detrimental role, depending on the METH regime/dose and brain region. Hence, repeated METH treatment leads to TNF-α overexpression attenuating METH-induced increase of extracellular striatal dopamine through the activation of vesicular dopamine uptake, which suggests a neuroprotective role for TNF-α (Nakajima *et al.*, 2004). On the other hand, Sriram and collaborators (2006a) reported that TNF-α might contribute to degenerative changes induced by METH since the lack of TNF receptor 1 (TNFR1) and receptor 2 (TNFR2) provided protection in the striatum. Interestingly, recent reports demonstrate that certain pharmacological agents are effective in attenuating METH-induced neurotoxicity, via blockade of microglial activation, such as MK-801, dextromethorphan (Thomas & Kuhn, 2005b) and anti-inflammatory drugs (Asanuma *et al.*, 2003; Tsuji *et al.*, 2009).

Since the hippocampus seems to be particularly susceptible to METH, with the present study we aimed to clarify the effect of an acute high dose of METH on mice hippocampal glial cells and neurons, and to evaluate the changes in the pro-inflammatory cytokine TNF-α and their receptors. Moreover, an anti-inflammatory treatment was used in an attempt to prevent the toxic effects induced by METH.

3.3. Material and Methods

3.3.1. Animals

Male wild-type C57BL/6J mice (3 months and 20-30g body weight) were used (Charles River Laboratories, Inc, Barcelona, Spain). The animals were housed under controlled environmental conditions [12 h light-dark cycle, at room temperature (RT) of 21±1ºC] with food and water available *ad libitum*. All experimental procedures were performed accordingly to the guidelines of the European Community for the use of animals in the laboratory (86/609/EEC) and the Portuguese law for the care and use of experimental animals (DL nº 129/92). The present study was approved by Foundation for Science and

Technology. To western blot studies mice were killed by decapitation, whereas to immunohistochemistry studies the animals were killed by perfusing transcardially a saline solution and paraformaldehyde 4% after anesthesia with an intraperitoneal injection of pentobarbital.

3.3.2. Drug treatments

The drug (α-methylphenethylamine methamphetamine) was synthesized in the Organic Chemistry Department, Faculty of Pharmacy, University of Porto, Portugal. Mice were injected intraperitoneally (i.p.) with a dose of 30 mg⁄kg of METH (dissolved in sterile 0.9% NaCl), and were killed at 1, 24h, 3 and 7 days post-drug administration. In parallel, another set of animals was administrated (i.p.) with 10 mg⁄kg indomethacin (INDO; Sigma-Aldrich, St Louis, MO, USA; dissolved in Tris–HCl 0.2 M, pH 8.2) 30 min prior to the injection of METH, and mice were killed at the same time-points abovementioned. Moreover, due to the fact that our study evaluated hippocampal alterations at different time-points and to exclude possible changes induced by manipulation and environment, we performed several controls as follows: mice were injected with 100 μl of sterile 0.9% NaCl or with 10 mg⁄kg INDO, and killed 1, 24h or 7 days post-injection. As there were no differences within the saline groups or within the INDO groups, we analyzed all the results and identified them as Control group or INDO group.

3.3.3. Western blot analysis

After appropriate treatment, mice were killed by decapitation and hippocampi were dissected on ice. The tissue was then homogenized in lysis buffer (50 mM Tris–HCl pH 7.4, 0.5% Triton X-100, 4°C) supplemented with a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μl/ml chymostatin, 1 μl/ml leupeptin, 1 μl/ml antipain and 5 μl/ml pepstatin A; all from Sigma). Protein concentration was then determined by the BCA method (Smith *et al.*, 1985) and stored at -80°C until further use. Total protein (Table 3.1) was separated by electrophoresis on sodium dodecyl

sulfate–polyacrylamide gel electrophoresis, transferred onto polyvinylidine difluoride membrane (Millipore, Madrid, Spain) and blocked with 5% non-fat milk or 4% bovine serum albumin for 1h at RT. Afterwards, the membranes were probed with the primary antibodies overnight at 4°C, as described in Table 3.1. Membranes were then washed, incubated for 1h at RT with alkaline phosphatase-conjugated secondary antibodies (1:20000; Amersham, GE Healthcare Life Science, USA), and visualized using ECF reagent (Amersham) on the Storm 860 Gel and Blot Imaging System (Amersham, GE Healthcare Life Science, Buckinghamshire, UK). Immunoblots were reprobed with β-actin antibody to ensure equal sample loading, and densitometric analyses were performed using the ImageQuant software.

3.3.4. Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (Sigma-Aldrich) and intracardially perfused with 10 ml of 0.01 M phosphate-buffered saline solution (PBS, in mM: NaCl, 137; KCl, 2.7; Na_2HPO_4, 4.3; KH_2PO_4, 1.47; pH 7.4) followed by 20 ml of 4% paraformaldehyde (PFA) in 0.01 M PBS, pH 7.4. The brains were removed and post-fixed overnight in 4% PFA solution at 4°C, followed by immersion in 20% sucrose in 0.01 M PBS over 24h at 4°C. Afterwards, the brains were cut into 30-μm coronal sections across the hippocampus, and slices were collected in 20% sucrose solution until used for free-floating immunohistochemistry. Hippocampal sections were rinsed in 0.01 M PBS (pH 7.4), blocked with 3% fetal bovine serum (FBS) + 1% Triton X-100 in 0.01 M PBS for 1h at RT, and incubated with the antibodies for 48h at 4°C. Astrocytes were visualized using a monoclonal antibody for glial fibrillary acidic protein (GFAP; 1:500; mouse anti-GFAP, Cy3 conjugated; ref. C9205 from Sigma-Aldrich), and microglial cells were labeled with rat anti-mouse CD11b antibody (1:500; ref. MCA71 from AbD Serotec) followed by incubation with Alexa Fluor 488 secondary antibody (1:200; ref. A11006 from Invitrogen) for 1h 30 min at RT. Sections were then washed, incubated with Hoechst 33342 (5 μg/ml; ref. 14533

from Sigma-Aldrich) for 5 min at RT in the dark, and mounted in Dako fluorescence medium (Dako North America).

Double-labeling for CD11b and TNF-a was performed as previously described (Clausen *et al.*, 2008). Briefly, sections were rinsed in 0.01 M PBS (pH 7.4), blocked with 10% FBS + 0.5% Triton X-100 in 0.01 M PBS for 30 min at RT, and incubated with rat anti-mouse CD11b antibody overnight at 4°C. Afterwards, brain sections were rinsed in PBS and incubated with Alexa Fluor 594 secondary antibody (1:200; ref. A11007 from Invitrogen) for 2h. From this point forward, the slices were protected from light. After washing with PBS, we incubated the sections for 24 h at 4°C with the antibody against TNF-α (1:100; ref. P350 from Endogen) diluted in 0.01 M PBS + 0.5% Triton X-100 containing 10% FBS. The following day, we rinsed the slices in PBS at RT, and incubated with the Alexa Fluor 488 secondary antibody (1:200; Invitrogen) for 3h at RT. Then, the sections were again rinsed, incubated with Hoechst 33342 (5 µg/ml; Sigma-Aldrich) and mounted using Dako fluorescence mounting medium (Dako North America). Images were recorded using an LSM 510 Meta Confocal microscope or an Axiovert 200 M fluorescence microscope (both from Carl Zeiss, Oberkochen, Germany).

3.3.5. Statistical analysis

The data are expressed as means ± SEM. Statistics were performed using one-way ANOVA followed by Dunnett's or Bonferroni's *post hoc* test, as indicated in the figure legends. Data were considered to be statistically different at $P < 0.05$.

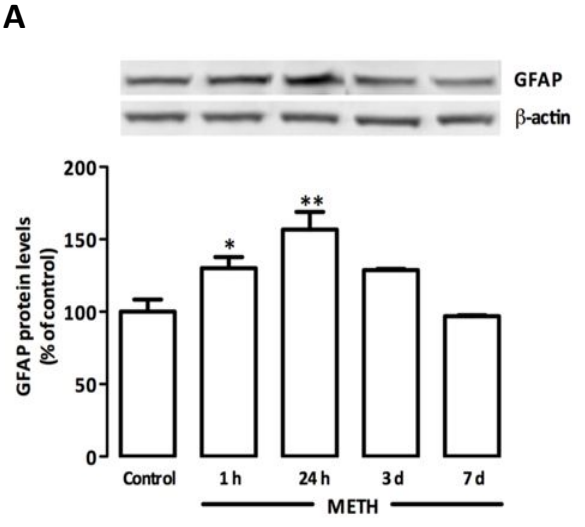
Table 3.1. *List of primary antibodies used in Western blot analysis*

Primary antibody	Molecular weight (kDa)	Protein concentration (μg/μl)	Dilution	Reference	Company
Rabbit anti-GFAP	55	1	1:20000	G9269	Sigma-Aldrich,
Mouse anti-Tuj-1	50	0.5	1:1000	A488-435L	Covance,
Rabbit anti-TNF-α	19	5	1:250	P350	Endogen, Thermo Scientific
Rabbit anti-TNFR1	55	20	1:200	sc-7895	St. Cruz Biotechnology
Rabbit anti-TNFR2	75	20	1:200	T1815	Sigma-Aldrich,
Mouse anti-SNAP-25	25	2.5	1:20000	S5187	Sigma-Aldrich
Mouse anti-syntaxin	39	2.5	1:10000	110011	Synaptic Systems,
Mouse anti-synaptophysin	37	2.5	1:5000	S5768	Sigma-Aldrich
Mouse anti-PSD-95	95	2.5	1:1000	Ab2723	Abcam
Mouse anti-tau	50	2.5	1:1000	#4019	Cell Signaling Technology
Rabbit anti-calbindin D28k	28	2.5	1:1000	#2136	Cell Signaling Technology
Rabbit anti-caspase 3	35/17	2.5	1:1000	#9662	Cell Signaling Technology
Mouse anti-β-actin	42	-	1:2000	A5441	Sigma-Aldrich

3.4. Results

3.4.1. METH induces hippocampal glial cells reactivity and alterations in the TNF system

It was recently proposed that gliosis frequently accompanies METH-induced brain toxicity (LaVoie *et al.*, 2004; Quinton & Yamamoto, 2006; Sriram *et al.*, 2006b; Simões *et al.*, 2008). Thus, in order to investigate the effect of an acute high dose of METH on glial cells, we evaluated the changes in GFAP protein levels. We observed that METH induced a significant increase in GFAP protein levels to 130.1 ± 7.6% of control or 156.7 ± 12.3% of control ($P < 0.05$, F = 5.36, d.f. = 16, n = 8) at 1 or 24h post-injection, respectively (Figure 3.1A), with no significant changes at 3 and 7 days post-administration (128.8 ± 0.8% and 96.9 ± 0.7% of control). These results were further corroborated by immunohistochemistry studies, as shown in Figure 3.1B where we can visualize a significant reactivation of astrocytes following 24h of METH administration in the hippocampus.



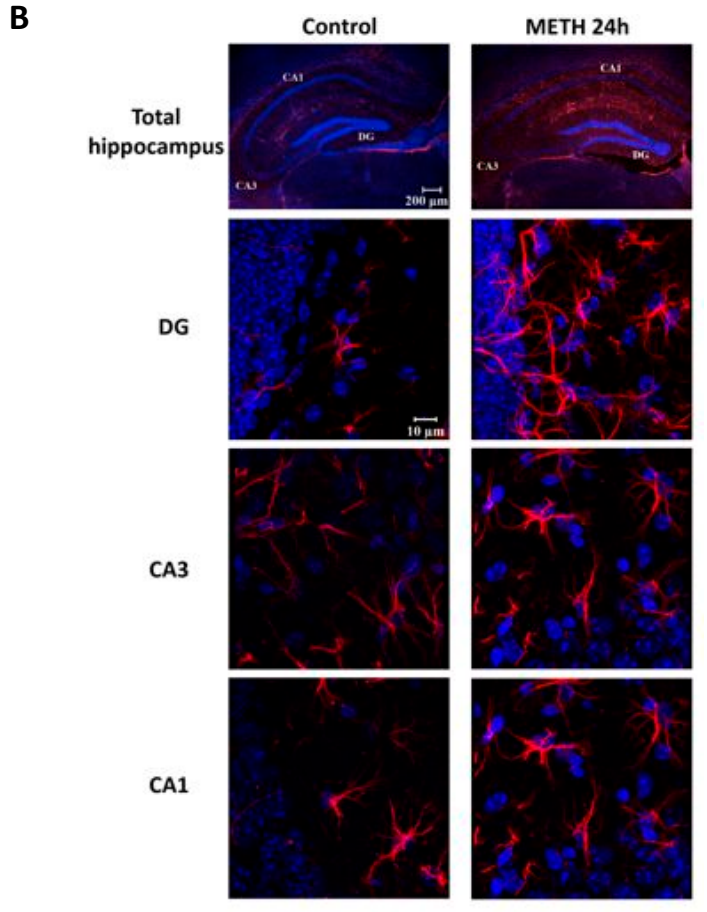


Figure 3.1. *Methamphetamine (METH) induced astrogliosis in the mice hippocampus. (A) Quantification of glial fibrillary acidic protein (GFAP) protein levels at 1, 24h, 3 and 7 days following a single high dose of METH (30 mg / kg) in total hippocampi extracts. Above the bars, representative western blots for GFAP (55 kDa) and β-actin (42 kDa) are shown. The results are expressed as mean percentage of control ± SEM of at least eight animals.* $^{*}P < 0.05$, $^{**}P < 0.01$ *– Dunnett's post-test, significantly different from control (saline; see previous page). (B) Representative fluorescence images of GFAP labeling in the total hippocampus and representative confocal images of astrocytes morphology in the dentate gyrus (DG), region inferior cornu ammonis (CA3) and region superior cornu ammonis (CA1) hippocampal subregions in control conditions and in the presence of METH (24h post-administration). Total brain sections (30 µm) were labeled with anti-GFAP, Cy3 conjugated (1:500, red), followed by incubation with Hoechst 33342 (blue). In total hippocampus images scale bar: 200 µm; hippocampal subregions images scale bar: 10 µm.*

Moreover, to determine if METH also causes activation of microglia, we examined the morphology of these cells using CD11b immunostaining, and we observed that microgliosis was evident just after 1h post-drug administration (Figure 3.2); however, this was more prominent at 24h in the three hippocampal subregions analysed [dentate gyrus (DG), region inferior cornu ammonis (CA3) and region superior cornu ammonis (CA1)], and remained activated at 7 days post-METH (Figure 3.2).

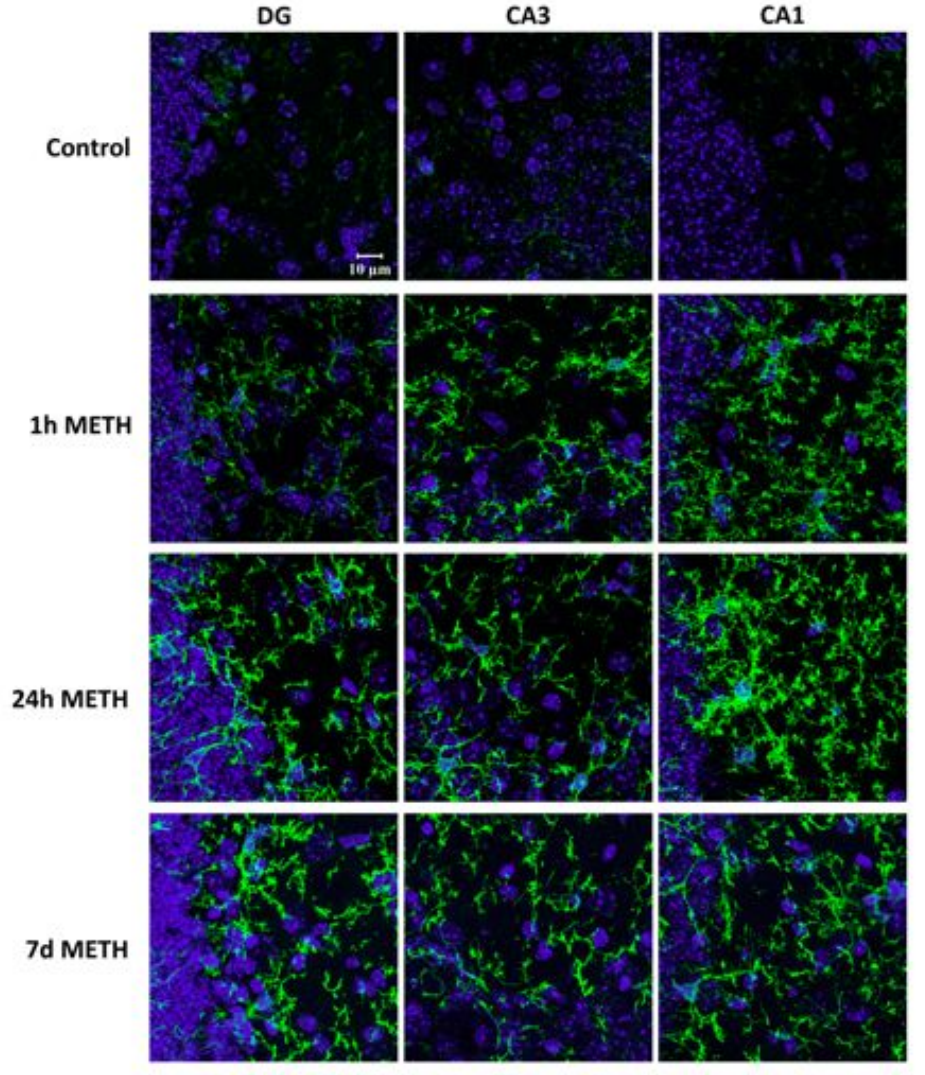


Figure 3.2. *Activation of microglial cells in the mouse hippocampus induced by methamphetamine (METH). Immunofluorescent labeling with anti-CD11b (1:500, green) demonstrated that activated microglia are present in the dentate gyrus (DG), region inferior*

cornu ammonis (CA3) and region superior cornu ammonis (CA1) subregions at 1, 24h and 7 days after an acute treatment of METH. Total brain sections (30 μm) were also stained with Hoechst 33342 (blue). All images were taken using a confocal microscope. Scale bar: 10 μm.

It is known that astrocytes and microglial cells are the main source of pro-inflammatory cytokines in the brain (Piehl & Lidman, 2001; McGeer & McGeer, 2002). Moreover, we have previously demonstrated that an acute METH treatment induces an early increase of TNF-α mRNA levels in the mice hippocampus (Gonçalves *et al.*, 2008). Thus, to further investigate the possible involvement of the TNF system in conditions of METH insult, we analysed the changes of TNF-α, TNFR1 and TNFR2 protein levels. Immunoblotting assay showed that at 7 days post-METH a significant increase of TNF-α protein levels occurs (151.9 ± 4.5% of control, $P = 0.008$, $F = 6.33$, d.f. = 15, n = 6; Figure 3.3A), with no significant changes after 1, 24h and 3 days (91.4 ± 8.7%, 90.0 ± 10.6% and 116.1 ± 2.9% of control, respectively). At the cellular level, the TNF-α immunoreactivity was detected in activated microglial cells after 7 days of METH administration, as shown by dual-staining for CD11b and TNF-a (Figure 3.3B).

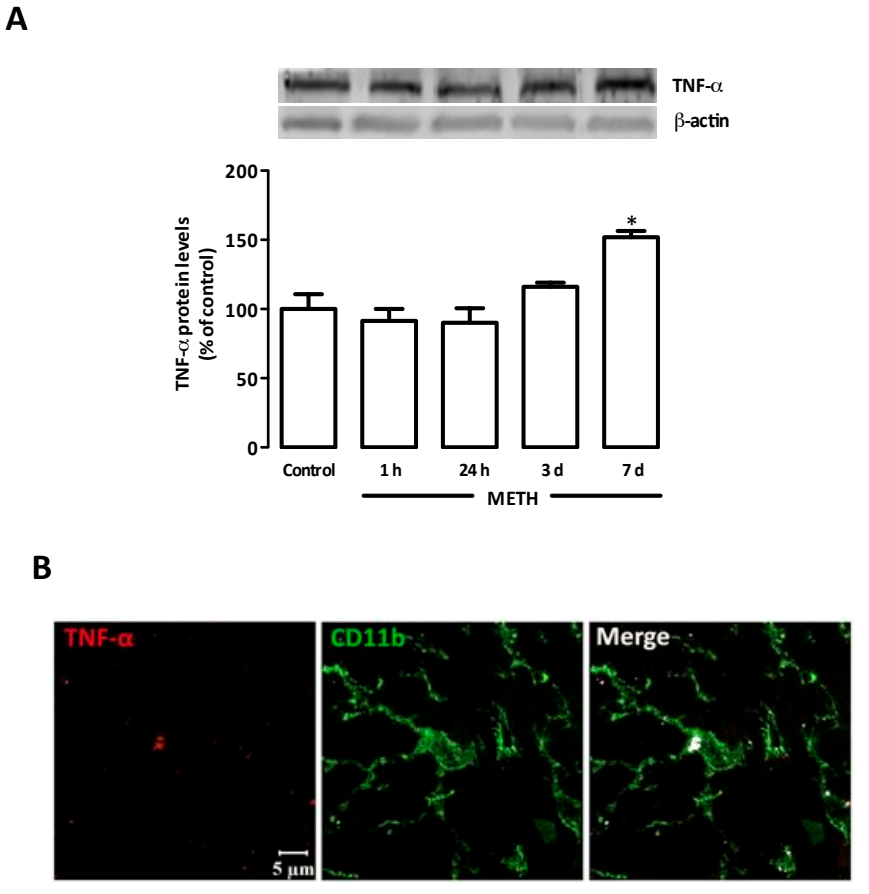


Figure 3.3. *Increase of tumor necrosis factor (TNF)-α protein levels by methamphetamine (METH). (A) Quantification of TNF-α protein levels at 1, 24h, 3 and 7 days post-METH administration Above the bars, representative western blots images of TNF-α (19 kDa) and β-actin (42 kDa) are shown. The results are expressed as mean percentage of control ± SEM of at least six animals.* $^{*}P < 0.05$ *– Dunnett's post-test; significantly different from control (saline). (B) Representative images of the double-staining for TNF-α (red, 1:100) and/or CD11b (green, 1:500) show that TNF-α immunoreactivity was detected in activated microglial at 7 days post-METH (white staining). The images were recorded using a confocal microscope. Scale bar: 5 µm.*

Regarding TNF receptors, we observed that METH induced an upregulation of TNFR1 protein levels after 1 and 24h to 204.1 ± 24.5% and 370.1 ± 39.5% of control, respectively ($P < 0.05$, F = 19.31, d.f. = 23, n = 4; Figure 3.4A). However, no significant differences were detected in TNFR2 protein levels following METH injection (Figure 3.4B).

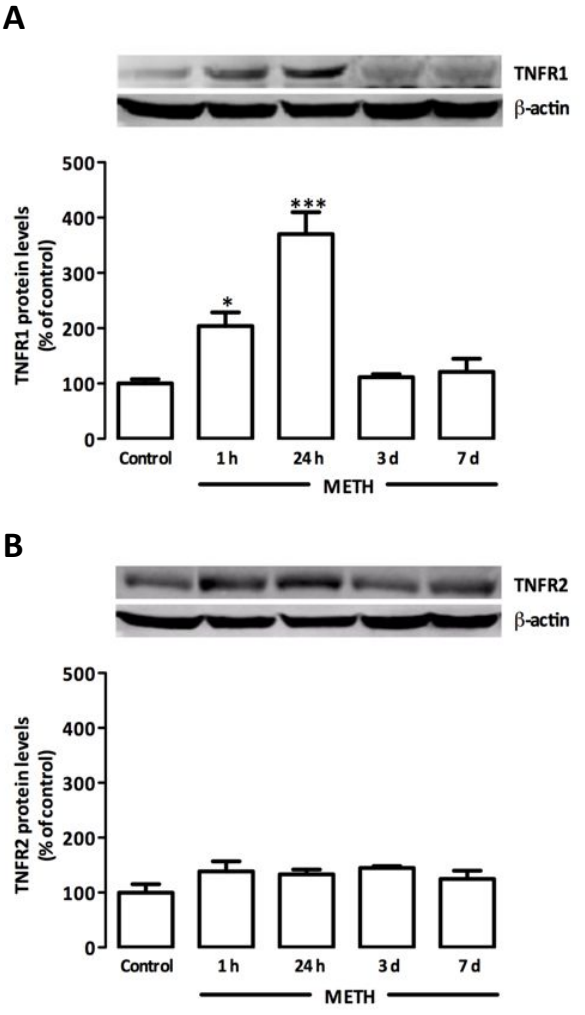
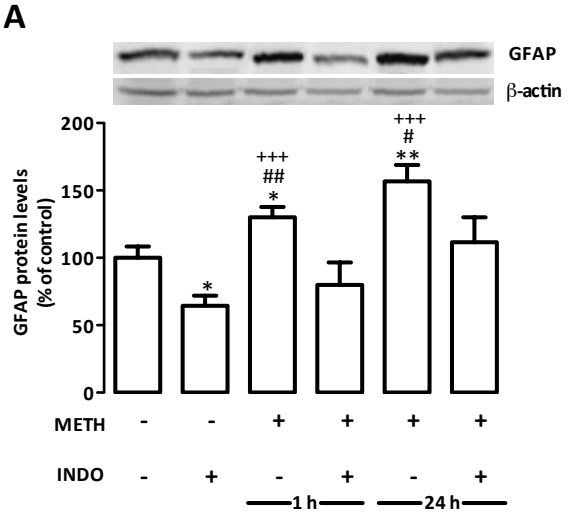


Figure 3.4. *Methamphetamine (METH) induced an increase in tumor necrosis factor receptor 1 (TNFR1) protein levels. Quantification of (A) TNFR1 and (B) tumor necrosis factor receptor 2 (TNFR2) protein levels in the mice hippocampus showing an increase in the TNFR1 levels at 1 and 24h post-METH administration without affecting the basal levels of TNFR2. Above the bars, representative western blot images of TNFR1 (55 kDa), TNFR2 (75 kDa) and β-actin (42 kDa) are shown. The results are expressed as mean percentage of control ± SEM of at least four animals.* $^{*}P < 0.05$, $^{***}P < 0.001$ *– Dunnett's post test; significantly different from control (saline).*

3.4.2. INDO prevents METH-induced neuroinflammation

Because gliosis and the consequent production of TNF-α following METH treatment are features of an inflammatory process, we further evaluated if the prior administration of INDO, which is a non-steroidal anti-inflammatory drug (NSAID), could prevent or attenuate METH-induced astrogliosis, microglia activation and alterations in the TNF system. We were able to demonstrate that pre-treatment with INDO prevents astrogliosis, as at 1 and 24 h post-METH the GFAP protein levels were similar to control (79.9 ± 16.7% and 111.5 ± 18.6% of control, respectively; Figure 3.5A). Furthermore, per se INDO induced a down-regulation of GFAP protein levels to 64.5 ± 7.4% of control (P = 0.03, F = 8.26, d.f. = 32, n = 4; Figure 3.5A). Regarding microglia, we observed that INDO prevented METH-induced microgliosis (data not shown), as the morphology was identical to control conditions. Therefore, we also concluded that INDO could prevent both the increase of TNF-α (83.3 ± 21.1% of control; Figure 3.5B) and TNFR1 protein levels (123.0 ± 15.3% and 103.5 ± 16.0% of control, at 1 and 24h post-METH, respectively; Figure 3.5C).



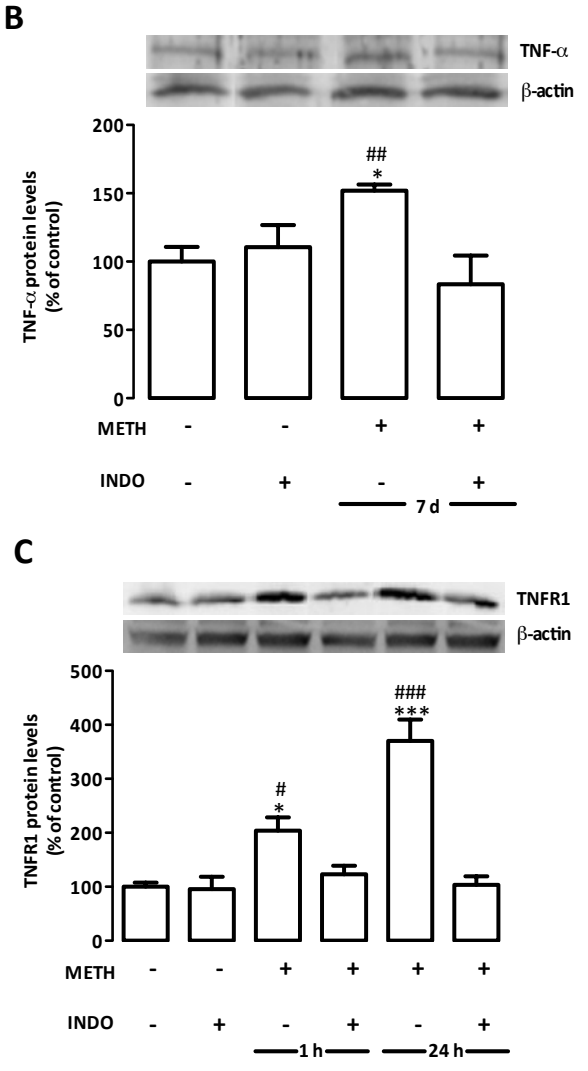


Figure 3.5. *Indomethacin (INDO) prevented methamphetamine (METH)-induced neuroinflammation in the mouse hippocampus. western blot analysis showed that pre-treatment with INDO (10 mg/kg) completely abolished the changes induced by METH on: (A) glial fibrillary acidic protein (GFAP; see previous page); (B) tumor necrosis factor (TNF)-α; and (C) tumor necrosis factor receptor 1 (TNFR1) protein levels. Above the bars, representative Western blot images are shown. The results are expressed as mean percentage of control ± SEM of at least four animals.* $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *– Dunnett's post-test; significantly different from control (saline).* $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *– Bonferroni's post-test; statistical significance when compared with METH plus INDO of the respective METH treatment group (1, 24h or 7 days).* $^{+++}P < 0.001$ *– Bonferroni's post-test; statistical significance when compared with INDO alone.*

These data clearly demonstrate that METH induces a neuroinflammatory process in the mice hippocampus, characterized by glial reactivity and an increase in the levels of TNF-α and TNFR1 and that an anti-inflammatory drug is able to prevent these alterations.

3.4.3. METH leads to hippocampal neuronal dysfunction

Several studies have demonstrated that both the reactivity of glial cells and upregulation of TNF-α can lead to cell death (Sriram & O'Callaghan, 2007; McCoy & Tansey, 2008). Thus, to investigate if METH induces cell death under the present experimental conditions, caspase-3 protein levels were assessed in total hippocampal lysates. However, we observed that METH treatment had no significant effect in the basal pro-caspase-3 protein levels (Figure 3.6), and western blot analysis failed to show the p17 subunit that corresponds to the activated caspase-3. Moreover, we also evaluated METH-induced apoptosis and neurodegeneration by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay and Fluor Jade C staining, respectively, but we did not observe differences between control conditions and METH treatment at 1, 24h, 3 and 7 days (data not shown). All together, these observations show that the METH administration paradigm used in the present study (acute high dose of METH) does not induce cell death in the mice hippocampus.

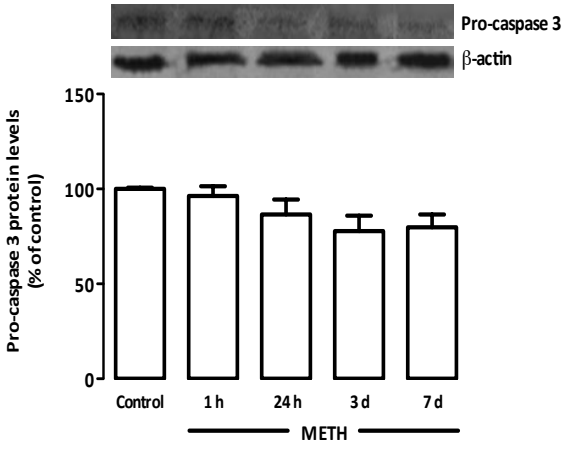


Figure 3.6. *Methamphetamine (METH) did not induce cell death by apoptosis in the mice*

hippocampus. Western blot analysis showing that a single high dose of METH (30 mg/kg) did not change protein levels of pro-caspase 3. Above the bars, representative western blot images of pro caspase 3 (35 kDa) and β-actin (42 kDa) are shown. The band corresponding to the activated caspase 3 (17 kDa) was not detected. The results are expressed as mean percentage of control ± SEM of at least four animals.

In fact, these results are in agreement with others previously published by our group (Simões *et al.*, 2007), where we showed that an acute or escalating dose administration of METH did not induce neurodegeneration in the rat hippocampus. However, we did observe alterations in N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor protein levels, and a mnemonic deficit in the rats acutely treated with the drug (Simões *et al.*, 2007). Thus, we hypothesized that METH could induce neuronal dysfunction, and in order to disclose how METH influences hippocampal neurons, we evaluated the possible changes in neuronal-specific class III beta-tubulin (Tuj-1) protein levels, a cytoskeletal neuronal marker. By Western blotting, we verified that METH induced a decrease in Tuj-1 protein levels after 24h (49.7 ± 3.5% of control), 3 days (61.8 ± 1.6% of control) and 7 days (62.4 ± 5.7% of control; $P < 0.001$, $F = 12.64$, d.f. = 33, n = 7; Figure 3.7A), and these results were confirmed by Tuj-1 immunostaining in hippocampal slices (data not shown). Likewise for GFAP protein levels and CD11b immunoreactivity, INDO pretreatment was able to prevent a METH-induced decrease in Tuj-1 protein levels to values similar to control at 24h, 3 and 7 days (102.8 ± 11.1%, 118.6 ± 21.5% and 96.7 ± 10.1% of control, respectively; Figure 3.7B).

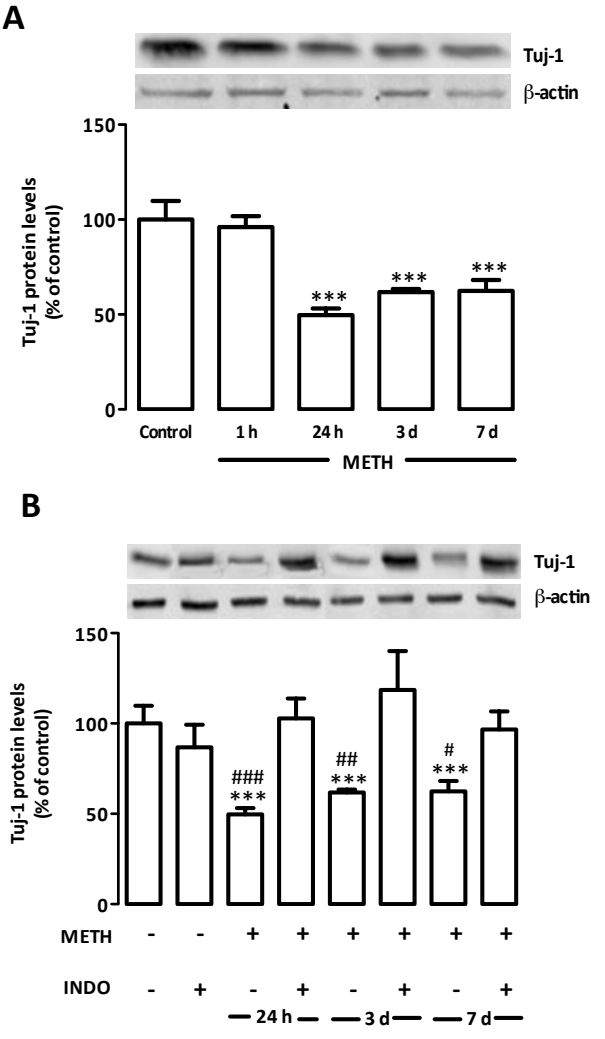


Figure 3.7. *Administration of methamphetamine (METH) led to neuronal-specific class III beta- tubulin (Tuj-1) downregulation. (A) Western blot analysis demonstrated that METH decreased the protein levels of Tuj-1 at 24h, 3 and 7 days after administration. (B) Indomethacin (INDO) pre-treatment prevented a METH-induced decrease in Tuj-1 protein levels. Above the bars, representative western blot images of Tuj-1 (50 kDa) and β-actin (42 kDa) are shown. The results are expressed as mean percentage of control ± SEM of at least seven animals.* $^{***}P < 0.001$ *– Dunnett's post-test; significantly different from control (saline).* $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *– Bonferroni's post-test; statistical significance when compared with METH plus INDO of the respective METH treatment group (24h, 3 or 7 days).*

To further support our hypothesis of neuronal dysfunction, we also evaluated the changes in the levels of several proteins that constitute the

exocytotic machinery, namely, syntaxin, synaptosome-associated protein of 25 000 Da (SNAP-25), synaptophysin and postsynaptic density-95 (PSD-95). Regarding presynaptic proteins, we observed that METH treatment induced a significant decrease of syntaxin-1 protein levels after 3 and 7 days to 77.4 ± 5.3% and 53.0 ± 2.6% of control, respectively ($P < 0.05$, F = 17.49, d.f. = 18, n = 4; Figure 3.8A); however, with no alterations in SNAP-25 levels (Figure 3.8B). Regarding synaptophysin, a synaptic vesicular marker, we could observe an increase in its protein levels to 154.7 ± 9.5% and 162.9 ± 10.1% of control ($P < 0.05$, F = 8.30, d.f. = 19, n = 5) at 1 and 24h, respectively, post-METH administration (Figure 3.8C), but no changes were observed concerning PSD-95, a postsynaptic protein (Figure 3.8D).

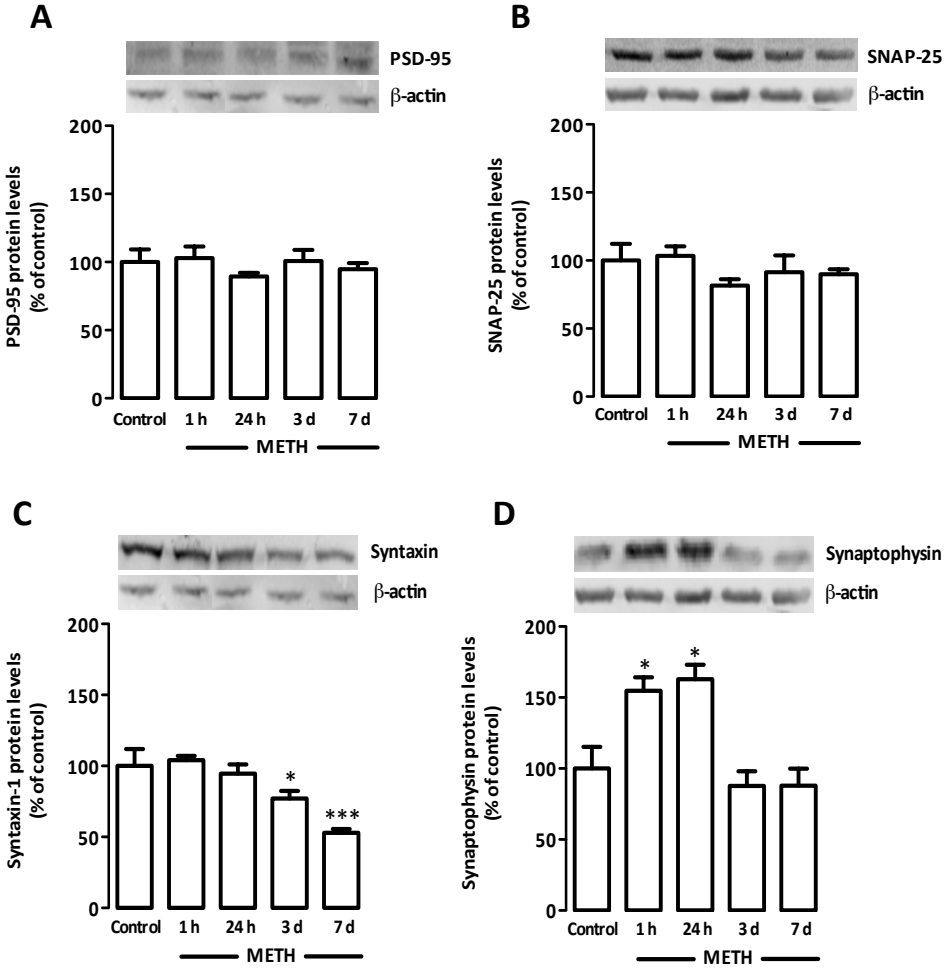


Figure 3.8. *Alterations in syntaxin and synaptophysin protein levels induced by*

methamphetamine (METH). (A) Western blot analysis indicated that METH induced a decrease in syntaxin protein (39 kDa) levels 3 and 7 days post-injection. (B and D) No alterations were detected in synaptosome-associated protein of 25 000 Da (SNAP-25; 25 kDa) and postsynaptic density-95 (PSD-95; 95 kDa) protein levels following METH administration. (C) Quantification of synpatophysin (37 kDa) protein levels showed its increase at 1 and 24h after METH treatment. Above the bars are shown the representative western blot images of blotting assay. β-actin (42 kDa) was used as loading control. The results are expressed as mean percentage of control ± SEM of at least four animals. $^{*}P < 0.05$, $^{***}P < 0.001$ *– Dunnett's post-test; significantly different from control (saline).*

To verify if METH also affects other neuronal functions, we also analysed the protein levels of calbindin D28k, a calcium-binding protein, and tau, a microtubule-associated protein. Thus, the administration of a high dose of METH caused a significant down-regulation of calbindin D28k protein levels, at 1h (75.2 ± 4.0% of control), 24h (69.3 ± 5.6% of control), 3 days (61.0 ± 7.6% of control) and 7 days (59.6 ± 4.9% of control; $P < 0.01$, F = 7.97, d.f. = 24, n = 6; Figure 3.9A). Concerning tau protein, we concluded that METH induced a significant decrease of its levels only after 7 days (43.6 ± 8.4% of control; $P = 0.0001$, F = 11.90, d.f. = 26, n = 6; Figure 3.9B). Moreover, we evaluated the effect of INDO pretreatment in METH-induced alterations in syntaxin, synaptophysin, calbindin D28k and tau levels, and we observed that, using this specific protocol of administration, INDO did not prevent these alterations (data not shown). Taken together, these data suggest that an acute administration of METH induces neuronal dysfunction, characterized by changes in several neuronal proteins.

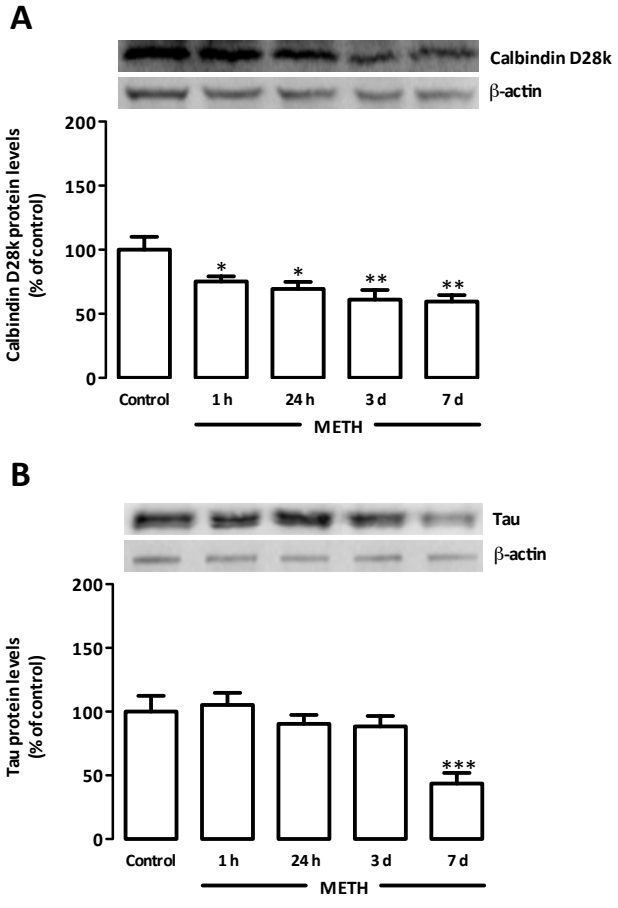


Fig. 3.9. *Downregulation of protein levels of calbindin D28k and tau following acute treatment of methamphetamine (METH). (A) Western blot analysis demonstrated that the levels of calbindin D28k decreased at 1, 24h, 3 and 7 days after the insult, whereas (B) METH induced a late reduction of tau protein levels post 7 days. Above the bars are shown the representative western blot images of calbindin D28k (28 kDa) and tau (50 kDa), and β-actin (42 kDa). The results are expressed as mean percentage of control ± SEM of at least six animals.* $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *– Dunnett's post-test; significantly different from control (saline).*

3.5. Discussion

Several studies have demonstrated that human METH abusers show a persistent reduction of dopaminergic terminals, as well as a reduction in dopamine transporters, which augment the risk for the development of

parkinsonism (McCann *et al.*, 2008). Moreover, Thompson et al. (2004) showed that chronic METH abuse causes a selective pattern of cerebral deterioration, including in the hippocampus, which contributes to impaired memory performance. Recently, Sekine et al. (2008) reported an increase of activated microglia in the brain of human METH abusers. Indeed, a repeated in vivo METH treatment induced a consistent and robust activation of microglial cells in mouse striatum (LaVoie *et al.*, 2004) and a significant increase in GFAP levels in several brain regions, namely striatum, cortex and hippocampus (Pubill *et al.*, 2003; Narita *et al.*, 2005, 2008; Simões *et al.*, 2007). Reactive gliosis is the most prominent response to diverse forms of CNS injuries, and METH administration is not an exception. In fact, the presence of reactive astrocytes has been used as a marker of neurotoxicity after METH chronic treatment (O'Callaghan & Miller, 1994). Furthermore, it is known that, in a neuropathological process microglia activation precedes or accompanies astrogliosis (Iravani *et al.*, 2005; Herber *et al.*, 2006). Accordingly, in the present study, we clearly observed that an acute high dose of METH induced an activation of astrocytes and microglia in the mouse hippocampus. Interactions that occur between reactive astrocytes and activated microglia following CNS injury, in particularly after a single high dose of METH, as well as their meaning for neuroregeneration and neurodegeneration are complex and remain unknown. However, in vitro studies demonstrated that microglial activation enhances the astroglial resistance to oxidative stress (Armbrust & Rohl, 2008) or induces an increase of IL-6 release that, in turn, improves the reactivity of astrocytes, which together promoted a neuroprotective mechanism (Eskes *et al.*, 2002).

When activated by an insult, glial cells, besides morphological alterations, get the capacity to produce and release high levels of pro-inflammatory cytokines (Olsson *et al.*, 1994; Piehl & Lidman, 2001; McGeer & McGeer, 2002), which are important mediators of inter-glial communications (Streit *et al.*, 1999). In normal physiological conditions, brain TNF-α levels are low but increase in a variety of CNS pathologies, such as trauma (Hua *et al.*, 2006), ischemia (Tuttolomondo *et al.*, 2008), multiple sclerosis (Centonze *et al.*, 2009)

and temporal lobe epilepsy (Lehtimaki *et al.*, 2003). Our group has previously demonstrated that METH induces a rapid increase of TNF-α mRNA levels in the mice hippocampus (Gonçalves *et al.*, 2008), and in the present study we further evaluated possible changes in protein levels of TNF-α and its receptors. We observed that TNF-α and TNFR1 protein levels significantly increased post-METH treatment. Moreover, we also showed that TNF-α is present in the microglia, which suggests that, in our model, these cells have a fundamental role in the production of this pro-inflammatory cytokine. Accordingly, several recent studies demonstrated that METH induces alterations in TNF-α mRNA and protein levels in the striatum, nucleus accumbens, hippocampus and amygdala (Nakajima *et al.*, 2004; Sriram *et al.*, 2006b; Lai *et al.*, 2009). Yet, Nakajima et al. (2004) verified that TNF-α is also expressed in the neurons on the nucleus accumbens after METH administration. Furthermore, the study of Lai et al. (2009) showed that TNF-α protein levels in mice striatum were up-regulated after 3 days of METH treatment, which corroborate our data as we also observed that an acute dose of METH led to a late increase of this cytokine, and not to an early upregulation as observed in other neuropathologies (Kolesnick & Golde, 1994; Mattson *et al.*, 1997).

Based on several studies, TNF-α can play an important role in sustaining and modulating neurodegeneration events, such as progression of multiple sclerosis or Parkinson's disease (for review see McCoy & Tansey, 2008), or in promoting pro-survival signaling and cellular differentiation (for review see Figiel, 2008). Indeed, regarding AMPA receptor-mediated excitotoxicity, a high dose of TNF-α potentiates neurodegeneration whereas a low concentration had a neuroprotective effect (Bernardino *et al.*, 2005b). It is well known that METH induces an increase in striatal extracellular dopamine levels together with a loss of tyrosine hydroxylase levels, and Nakajima et al. (2004) showed that the treatment with exogenous TNF-α attenuated all these METH effects in the striatum. Moreover, the same study demonstrated that a systemic administration of TNF-α inhibits the METH-induced hyperlocomotion,

evidencing the neuroprotective role of this pro-inflammatory cytokine (Nakajima *et al.*, 2004). On the other hand, Sriram et al. (2006a) associated the striatal degenerative changes observed 12h after administration of METH with an increase of TNF-α mRNA levels, as they showed that deficiency of both TNFRs protected against METH-induced dopaminergic neurotoxicity. However, another study from the same authors (Sriram *et al.*, 2006b) showed that an acute treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induces hippocampal TNF-α mRNA overexpression 12h after administration, and that this cytokine had a neuroprotective effect in the hippocampus. In fact, very recently it was suggested that microglia-derived TNF-α is essential for neuroprotection because it allows microglia to promote survival of endangered neurons, rather than neuronal loss, and regulates their own survival and functional profile (Lambertsen *et al.*, 2009). So, all together these studies clearly show that TNF-α can exert a dual role depending on the insult and brain region. Furthermore, the TNF-α pleiotropic action is mediated through two distinct cell surface receptors, TNFR1 and TNFR2, and both are expressed by neurons and glial cells (Kinouchi *et al.*, 1991). To determine whether this pro-inflammatory cytokine will exert a beneficial or detrimental effect on the CNS, it is important to take in consideration the distribution, the state of activation and the downstream effectors of TNF receptors (McCoy & Tansey, 2008). Our results demonstrated that an acute injection of METH induces an increase of TNFR1 protein levels without causing alterations in TNFR2. Normally, TNFR1 is related to activation of apoptosis, because it contains a cytoplasmatic death domain (Tartaglia *et al.*, 1993b), but it can also induce the activation of nuclear factor kappa-B, that in turn initiates pro-survival signaling, cellular proliferation and cytokine production (McCoy & Tansey, 2008). In the present work, analyzing together the changes in the TNF system, we demonstrated that METH induced an early upregulation of TNFR1 (at 1 and 24h) and a late increase of TNF-α (only after 7 days). Although we did not investigate the underlying mechanisms responsible for this different temporal profile, others have also observed a discrepancy within the TNF system. Indeed, Rosenzweig et al. (2007) showed

that, following a middle cerebral artery occlusion, the cortical protein levels of TNFR1 increased earlier (after 3h) than TNF-α levels (after 24h), suggesting a neuroprotective role for enhanced levels of endogenous TNFR1. On the contrary, Harry et al. (2008) demonstrated that treatment with trimethyltin (TMT) induced an upregulation of mRNA levels of TNF-α after 18h and an increased level of TNFR1 at 72h in the mice hippocampus. Interestingly, they concluded that DG and the CA1 sub-regions respond differently following TMT. Indeed, they observed an elevation of TNF-α levels within the DG that occurred earlier and was higher than that in the CA region, suggesting the possibility of a threshold requirement for TNF-α levels to initiate receptor internalization and activation.

The astrogliosis, microglia activation and production of TNF-α are features of an inflammatory process. Thus, we hypothesized that a NSAID, such as INDO, could prevent or at least decrease the METH- induced neuroinflammation in the mice hippocampus. INDO is a non-selective inhibitor of cyclooxygenase (COX)-1 and -2 that participate in prostaglandin synthesis, which are lipid metabolites involved in inflammation (Farooqui *et al.*, 2007), and due to its lipophilic nature INDO readily crosses the blood–brain barrier (BBB; Parepally *et al.*, 2006). Zhang et al. (2007) verified that during the earlier stages of METH-related neurotoxicity in the striatum, the cells that contain COX-2 are particularly affected, and demonstrated that COX-1 is less likely to be involved in METH neuropathologies. In addition, it was observed that METH induced an increase in COX-2 protein levels, and COX-2 knockout mice treated with METH were resistant to its nocive effects (Thomas & Kuhn, 2005a). Hence, COX-2 seems to be an obligatory factor in METH toxicity. Here, we reported for the first time that a pretreatment with INDO is able to prevent METH-induced neuroinflammation in the mice hippocampus. Both astrocytes and microglial cells react to an insult with production and secretion of pro-inflammatory cytokines, namely TNF-α, which maintain the inflammatory process by a number of mechanisms, including the activating of the COX pathway (for review see Farooqui *et al*., 2007). Therefore, when we pre-treated mice with INDO, we

blocked the COX signaling and, as a result, the effect of inflammatory mediators produced by glial cells was compromised. In this manner, we conclude that the prior INDO treatment affords protection against the inflammatory process triggered by a high dose of METH in the hippocampus.

Beyond astrocytes and microglia, we also investigated the effect of METH on hippocampal neurons. We observed a significant decrease in Tuj-1 protein levels that was prevented by INDO pretreatment. Tuj-1 is a neuron-specific cytoskeletal protein, and its expression can provide a unique insight into the neuronal response to insults and alterations in the composition of the neuronal cytoskeleton (Zhu *et al.*, 2007). Although some authors use Tuj-1 as a marker to identify immature (young) neurons (Seri *et al.*, 2004), others use it as a general marker for neurons (Gould *et al.*, 2001; Tan *et al.*, 2009). Moreover, some studies reported that after a hippocampal insult, specifically stress, the immunoreactivity of Tuj-1 decreased, which can mean morphological changes in neuronal structures (Bu & Lephart, 2005; Zhu *et al.*, 2007). The decrease of Tuj-1 levels, as well as the absence of cell death at all time-points analysed, which is in accordance with previous studies (Pereira *et al*., 2006; Simões *et al*., 2007), lead us to suggest that METH induces neuronal dysfunction in the mice hippocampus. This hypothesis was further corroborated with the observation of significant changes in synaptic proteins, namely syntaxin-1 and synaptophysin, in calbindin D28k, which is a calcium-binding protein, and also in a microtubule-associated protein abundant in neurons (tau). In agreement with our results, in multiple sclerosis and cerebral ischemia models it was demonstrated that immediately after inflammatory damage occurred microglial activation and TNF-α release that, in turn, induced changes in glutamate transmission and, consequently, alteration in synaptic transmission (Centonze *et al.*, 2009; Kriz & Lalancette-Hebert, 2009). Moreover, the changes in the levels of immune molecules in the brain, namely pro-inflammatory cytokines, in response to insults may lead to unexpected dysfunctions due to their direct neural actions and⁄or disruption of their normal adaptive role in the brain (Stellwagen & Malenka, 2006). In fact, it was suggested that, by modulating TNF-α levels, glia

actively participates in the homeostatic activity-dependent regulation of synaptic connectivity (Stellwagen & Malenka, 2006).

It is known that METH consumption causes cognitive deficits (Thompson *et al*., 2004; Simões *et al*., 2007) that have been correlated with both dysfunction of monoaminergic systems (Belcher *et al.*, 2005; Castner *et al.*, 2005) and alterations in glutamatergic systems (Simões *et al*., 2007; Gross & Marshall, 2009). So, it is plausible to suggest that dysfunction of synaptic transmission underlies these nocive effects of METH. In fact, we showed alterations in proteins implicated in synapse and axonal transport, such as a decrease in syntaxin-1 protein levels. It is known that this protein plays an important role in learning behavior in vivo (Takasusuki *et al.*, 2007). Indeed, the formation of long-term potentiation is associated with an increase in levels of syntaxin-1 that occur with the augment of depolarization-induced glutamate release, which in turn leads to the propagation of synaptic plasticity (Helme-Guizon *et al.*, 1998). On the contrary, after an acute METH treatment we verified an up-regulation in hippocampal synpatophysin levels. Previously, Rademacher et al. (2006) reported that a high dose of amphetamine induced a significant increase in synpatophysin levels in the hippocampus, and demonstrated that this structural change had implications in the reward system. In fact, it is known that an augment of synpatophysin expression means an increase in the number of synaptic vesicles and, consequently, in the number of synapses (Calhoun *et al.*, 1996; Liu & Ju, 2001). Furthermore, we showed that METH decreases the expression of tau, an axonal transport protein, which suggests that METH induces alterations in the neuronal cytoskeleton, beyond synaptic alterations. Tau protein plays a role in regulating microtubule dynamics, axonal transport and neurite outgrowth, mainly during periods of high plasticity in the developing mammalian brain (for review, see(Johnson & Stoothoff, 2004)). Xie et al. (2002) demonstrated that tau protein levels were decreased in mouse ventral midbrain after a high dose of METH. Moreover, other authors demonstrated that METH induced proteolytic cleavage of tau protein in parallel with depletion of serotonin in the striatum, hippocampus and cortex (Wallace

et al., 2003). It is also known that the abnormal hyperphosphorylation of tau protein is associated with Alzheimer's disease (for review see Iqbal *et al.*, 2009). Indeed, this alteration in tau seems to be the result of increased release of pro-inflammatory cytokine levels by microglia (for review see Maccioni *et al.*, 2009). Furthermore, it was demonstrated that microglial-derived TNF-a induced the accumulation and aggregation of tau in neuritis (Gorlovoy *et al.*, 2009). In the present study, we clearly show that at 7 days after METH administration there was an increase and a decrease of TNF-a and tau protein levels, respectively. However, a direct correlation between these alterations induced by METH remains to be elucidated.

In conclusion, the present study reports that a single high dose of METH triggers a neuroinflammatory process in the mice hippocampus characterized by astrogliosis, microglial activation and alterations in the TNF system. We also demonstrated that METH induces neuronal dysfunction with alterations in the cytoskeleton and in both synaptic and axonal proteins, without evidences of cell death. Moreover, the pre-treatment with an anti-inflammatory drug was able to prevent neuroinflammation features induced by METH. Taken together, these data provide us with important information regarding the hippocampal dysfunction induced by METH and a new tool to minimize its nocive effects.

Chapter 4

Protective effect of tumor necrosis factor-alpha against methamphetamine-induced microglial and neuronal cell death

4.1. Abstract

It is well known that methamphetamine (METH) is neurotoxic and recent studies have suggested the involvement of neuroinflammatory processes in brain dysfunction induced by this drug of abuse. Indeed, glial cells seem to be activated in response to METH, but its effects on microglial cells are not fully understood. Moreover, it has been shown that cytokines, which are normally released by activated microglia, may have a dual role in response to brain injury. This led us to study the effect of METH on microglial cells and hippocampal neurons by looking to cell death and alterations of tumor necrosis factor-alpha (TNF-α) system, as well as to the role played by this pro-inflammatory cytokine. For that, we used the N9 microglial cell line and mouse hippocampal organotypic slice cultures, in which cell death was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and propidium iodide (PI) uptake. Alterations in microglial morphology and proliferation following METH treatment were also investigated by immunocytochemistry. In both *in vitro* models, the contents of TNF-α were quantified by enzyme-linked immunosorbent assay (ELISA) and changes in TNF receptor 1 (TNFR1) by western blot analysis. We concluded that METH induces microglia cell death in a concentration dependent manner (EC_{50} = 1 mM), and led to significant morphogical changes and decreased cell proliferation. Additionally, this drug increased TNF-α extracellular and intracellular levels, as well as their receptor protein levels, at 1h post-drug exposure in microglia or hippocampal cultures. Interestingly, the endogenous pro-inflammatory cytokine did not contribute to METH-induced microglial cell death. On the other hand, exogenous low concentrations of TNF-α had a protective effect against microglial and neuronal toxicity induced by METH. These data clearly show that TNF-α has a protective role against METH-induced microglial and hippocampal neurons cell death.

4.2. Introduction

Methamphetamine (METH) is a potent addictive psychostimulant drug that easily crosses the blood-brain barrier (BBB) and induces severe brain damage leading to neurological abnormalities and eventually to psychiatric disorders. Several studies have demonstrated that METH abusers reveal deficits in dopaminergic and serotonergic systems, hippocampal volume reduction, white-matter hypertrophy and microglia activation (Thompson *et al.*, 2004; McCann *et al.*, 2008; Sekine *et al.*, 2008). However, the underlying mechanisms of its toxicity remain to be fully determined. Nevertheless, oxidative stress (Wu *et al.,* 2007; Pubill *et al.,* 2008), excitotoxicity (Stephans & Yamamoto, 1994; Rocher & Gardier, 2001), mitochondrial dysfunction (Brown *et al.,* 2005; Wu *et al.,* 2007), and more recently microgliosis (Hebert & O'Callaghan, 2000; LaVoie *et al.*, 2004; Gonçalves *et al.*, 2010) are some features of METH neurotoxicity.

Recently, our group demonstrated that a single high dose of METH [30 mg/kg; intraperitoneal (i.p.)] triggered a neuroinflammatory response in the mice hippocampus characterized by the activation of microglia and production of pro-inflammatory cytokines, namely tumor necrosis factor-alpha (TNF-α) and interleukine-6 (IL-6; Gonçalves *et al.,* 2008, 2010). Accordingly, Thomas *et al*. (2004) showed that microglia activation represents an early step in METH-induced striatal dopamine (DA) or serotonin (5-HT) nerve terminal damage. However, the attenuation of microglia activation is not by itself sufficient to protect against METH-induced striatal dopaminergic neurotoxicity, and this lack of neuroprotection was shown to be due to the inability of minocycline to modulate TNF-α signaling (Sriram *et al.,* 2006a). Moreover, it was reported that METH reduced rat microglial cells viability simultaneously with the increase of IL-6 and TNF-α expression and production of both reactive oxygen species (ROS) and reactive nitrogen species (RNS), suggesting that both cytokines may participate in METH toxicity (Tocharus *et al.,* 2010). Despite these pieces of evidence, it remains to be clarified if neuroinflammation, and consequent pro-

inflammatory cytokines synthesis and release, is a cause or consequence of the neurotoxicity induced by METH.

TNF-α is a pleiotropic cytokine that can exert its action through TNF receptor 1 (TNFR1) and receptor 2 (TNFR2) (Kinouchi *et al.,* 1991). Moreover, this molecule is typically up-regulated in several CNS disorders, such as ischemia (Tuttolomondo *et al.,* 2008) and multiple sclerosis (Centonze *et al.,* 2009). Interestingly, some authors have pointed out a beneficial role for TNF-α, which seems to be dependent on the type of brain insult, as well as brain region affected (Figiel, 2008; McCoy & Tansey, 2008). Moreover, the activation of its receptors stimulates several signaling pathways, which will regulate cellular processes ranging from cell proliferation and differentiation to cell death (McCoy & Tansey, 2008). Indeed, it was showed that TNF-α was able to diminish the reduction of vesicular DA uptake in striatum caused by a repeated METH treatment, playing a neuroprotective role (Nakajima *et al.,* 2004). In contrast, other study reported that TNF-α is an obligatory factor to METH toxicity, since the lack of TNFR1 and TNFR2 provided neuroprotection in striatum (Sriram *et al.,* 2006a).

In the present study we aimed clarify the role of TNF-α under METH-induced microglial and neuronal toxicity.

4.3. Material and Methods

4.3.1. Microglial cell culture

The murine microglia cell line N9 (kindly provided by Prof. Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy) were cultured in RPMI (Gibco, Paisley, UK) supplemented with 5% fetal bovine serum (FBS; Gibco), 23.8 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), 30 mM d-glucose (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), and were maintained at 37°C, 95% air and 5% CO_2 in a humidified incubator. N9 cells were then seeded onto: 24-well plates

with $1.6x10^4$ cells/well for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and 5-bromo-2'-deoxyuridine (BrdU) assays and immunocytochemistry; 12-well plates with $5.6x10^4$ cells/well for enzyme-linked immunosorbent assay (ELISA); 6-well plates with $5x10^5$ cells/well for western blot analysis.

4.3.2. Mouse hippocampal organotypic slice cultures

Hippocampal slice cultures were prepared from 6-8 day-old wild type C57BL/6 mice according to the interface culture method, as previously described by us (Bernardino *et al.*, 2005b; Xapelli *et al.*, 2007; Xapelli *et al.*, 2008). Specifically, after sacrifice, mice brains were removed under sterile conditions and both hippocampi were isolated and were cut in transverse slices with 350-µm-thick using a McIlwain tissue chopper. The slices were placed in ice-cold Gey's balanced salt solution (GBSS; Sigma-Aldrich St. Louis, MO, USA) with 25 mM D-glucose (Merck, Darmstadt, Germany) and 0.01% Penicillin-Streptomycin (Invitrogen, Paisley, UK), and the excess of tissue was removed. Afterwards, individual slices were placed on porous (0.4 µm) insert membranes (Millipore, Madrid, Spain) with a total of six slices in each membrane, and transferred to six-well culture trays (Orange Scientific, Braine-l'Alleud, Belgium). Each well contained 1 ml culture medium composed of 50% Opti-MEM, 25% heat-inactivated horse serum, 25% Hank's balanced salt solution (HBSS) and 0.01% Penicillin-Streptomycin (all from Invitrogen), supplemented with 25 mM d-glucose (Merck). Cultures were maintained in an incubator with 5% CO2 and 95% atmospheric air at 37°C with medium change twice each week for the following two weeks. Before starting the experiments, culture medium was replaced by serum-free Neurobasal medium (Invitrogen), supplemented with 1 mM L-glutamine (Sigma-Aldrich), 2% B-27 supplement and 0.01% Penicillin-Streptomycin (Invitrogen).

All experiments were conducted according to the guidelines of the European Community for the use of animals in the laboratory (86/609/EEC) and the Portuguese law for the care and use of experimental animals (DL 129/92).

Moreover, all efforts were made to minimize pain or discomfort, and the number of animals used.

4.3.3. Assessment of cell death

4.3.3.1. TUNEL assay

N9 cells were incubated with increasing concentrations (0.1 - 4 mM) of METH [(+)-Methamphetamine hydrochloride, Sigma-Aldrich] for 24h followed by cell death evaluation. The present concentration range of METH was chosen based on previous *in vitro* studies (Aizenman *et al.*, 2010; Nopparat *et al.*, 2010; Tocharus *et al.*, 2010; Langsdorf & Chang, 2011). In order to confirm if METH leads to an apoptotic process, cells were co-incubated for 24h with 1 mM METH plus 25 μM z-Val-Ala-DL-Asp (OMe)-fluoromethylketone (Z-VAD-fmk; Calbiochem, Nottingham, U.K.) that was chosen based on prior works developed by our group (Bernardino *et al*., 2008; Bento *et al*., 2011).

To investigate the contribution of endogenous and exogenous TNF-α, N9 cells were co-incubated with 100 μg/ml TNF-α antibody (Upstate, Lake Placid, NY) (Bernardino *et al*., 2005b) or 1 ng/ml TNF-α (R&D systems, Abingdon, UK) (Bernardino *et al*., 2005b), respectively, plus 1 mM METH during 24h (Figure 4.1A). After the respective treatments, we collected the supernatant that contains the cells that detached from the bottom of the wells (dead or dying cells), and adherent cells (surviving cells) were trypsinized and added to the detached cells in order to obtain the whole population of cells. Then, microglial cells were fixed with 4% parafaraformaldehyde (PFA) and adhered to superfrost microscope slides (Thermo scientific, Menzel GmbH & Co KG, Braunschweig, Germany) by centrifugation (113 x*g*, 5 min; Cellspin I, Tharmac GmbH, Waldsolms, Germany). Apoptotic cell death was further evaluated by TUNEL assay (Roche Diagnostics GmbH, Mannheim, Germany). For that, cells were rinsed with 0.01 M phospato-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), permeabilized in 0.25% Triton X-100 for 30 min at room temperature (RT), and incubated with terminal deoxynucleotidyl transferase buffer for 1h at 37ºC in a humidified chamber.

Afterwards, N9 cells were washed in termination buffer (TB) buffer (300 mM NaCl and 30 mM sodium citrate) for 15 min and in 0.01 M PBS for 5 min. Incubation with fluorescein Avidin D (1:100; Vector Laboratories, Burlingame, CA, USA) was performed for 1h, followed by nuclei counterstaining with 5 μg/ml Hoechst 33342 (Sigma-Aldrich) for 5 min. The slides were mounted in Dako fluorescent medium (Dako North America, Carpinteria, USA) and fluorescent images for cell counts were recorded using Axiovert 200M fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

4.3.3.2. Propidium iodide uptake

Hippocampal slice cultures were pre-incubated for 24h with 1 ng/ml TNF-α (R & D Systems) (Bernardino *et al.*, 2005b), followed by co-exposure with 0.5 mM METH for more 24h (Gonçalves *et al.*, 2012) (Figure 4.1B). After treatments, neuronal cell death was assessed by monitoring the propidium iodide uptake [PI; 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide; Sigma-Aldrich] as previously reported (Bernardino *et al*., 2005b; Xapelli *et al*., 2007, 2008). Thus, 3h before starting experiments, 2 μM PI was added to the medium for determination of basal cellular uptake, and this concentration was also used in all subsequent medium changes. Cellular PI uptake was recorded by fluorescence microscopy (microscope Axioskop 2 Plus, Carl Zeiss, Göttingen, Germany) using a standard rhodamine filter and a digital camera (AxioCam HRc, Carl Zeiss). Before drug exposure (basal PI uptake) and at fixed time-points after drug exposure were taken digital fluorescent micrographs. PI uptake was quantified by delineating the *cornu ammonis* 3 (CA3) and *cornu ammonis* 1 (CA1) hippocampal subregions using NIH ImageJ 1.44 analysis software. The effect of treatments was assessed by subtracting the basal PI uptake value (day 0, non treated cultures) from the corresponding value after treatment (day 1 or day 2).

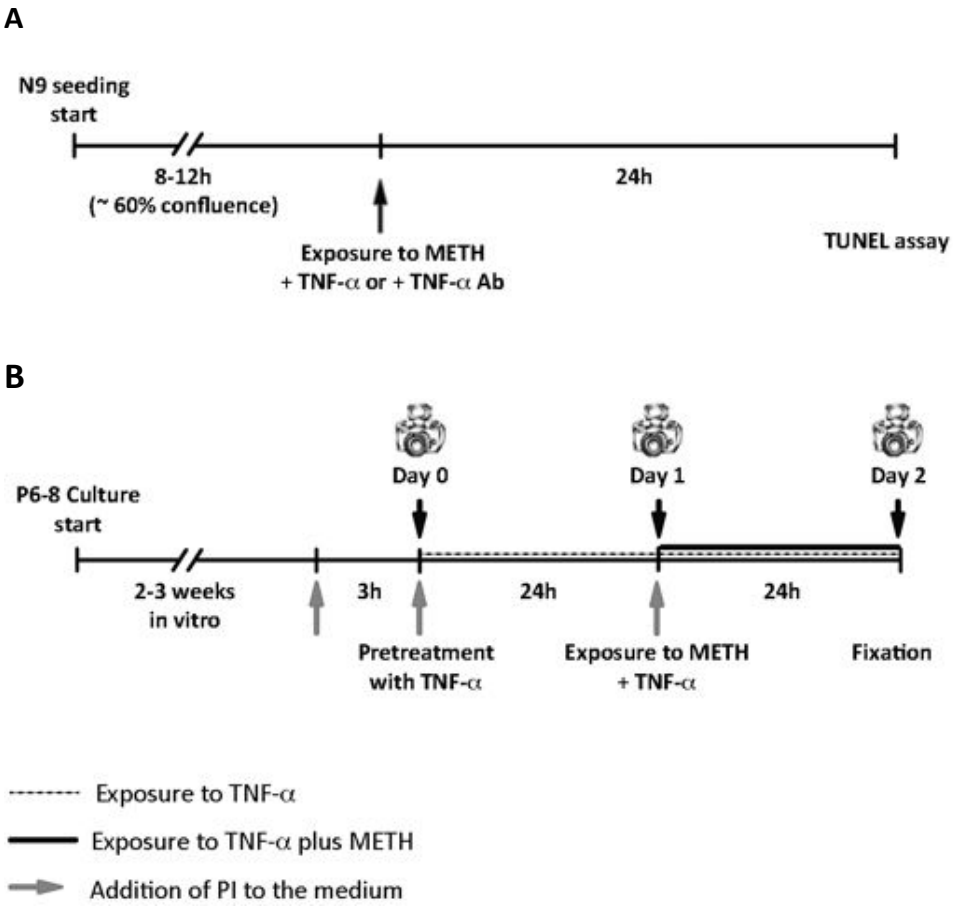


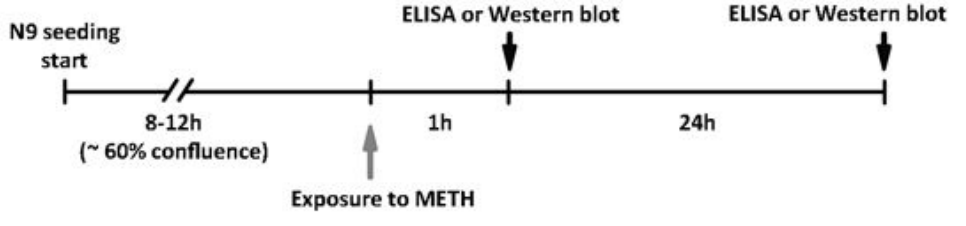
Figure 4.1. *Scheme of the experimental protocol to investigate the role of TNF-α under conditions of METH-induced toxicity on (A) N9 microglial cell line and (B) mouse hippocampal organotypic slice cultures. For details, see text.*

4.3.4. ELISA for TNF-α

A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystem®, Vienna, Austria) was used to evaluate the intracellular and extracellular contents of pro-inflammatory cytokine TNF-α. The microglial cells and mouse hippocampal organotypic cultures were treated with 1 mM and 0.5 mM METH, respectively, for 1h or 24h followed by ELISA assay (Figure 4.2A and B). The supernatant was removed and centrifuged for 15 min at 17968 x*g*, 4°C, and then cells were lysed using a specific buffer (pH 8.0) as follows: 150 mM NaCl, 10 mM Tris-HCl, 10% Triton X-100, 1 mM ethylenediamine tetraacetic

acid (EDTA) complemented by a protease inhibitor cocktail tablet (Roche Applied Sciences, Basel, Switzerland). Afterwards, microglial cells or hippocampal slices were sonicated, and protein concentration was determined by the bicinchoninic acid (BCA) method, and stored at -20°C until further use. ELISA assay was then performed according to manufacturers' instructions. Specifically, 96-well microtiter plates were coated with capture antibody (5 μg/ml), sealed and left overnight (ON) at 4°C. Then, wells were washed with 0.01 M PBS plus 0.05% Tween 20, blocked with assay buffer (0.01 M PBS + 0.5% BSA + 0.05% Tween 20), and left ON at 4°C. Samples (supernatants and cell lysates) and biotin-conjugated antibodies (1:1000) were added to all wells, and plate was incubated at RT for 2h on a microplate shaker (200 rpm). After washing, streptavidin-HRP (1:5000) was added and then kept once again at RT on a microplate shaker (200 rpm) for 1h. After washing, the tetramethylbenzidine (TMB) substrate solution (eBioscience, Vienna, Austria) was added to each well for 20 min at RT. The reaction was stopped by adding 1 M H_3PO_4, and the absorbance was measured with a microplate reader (Biotek, Synergy HT, Winooski, USA), using a sample wavelength fixed at 450 nm and a reference wavelength at 655 nm. A standard curve was used to calculate the respective extracellular (pg/ml) and intracellular (pg/mg of total protein levels) protein levels.

A



B

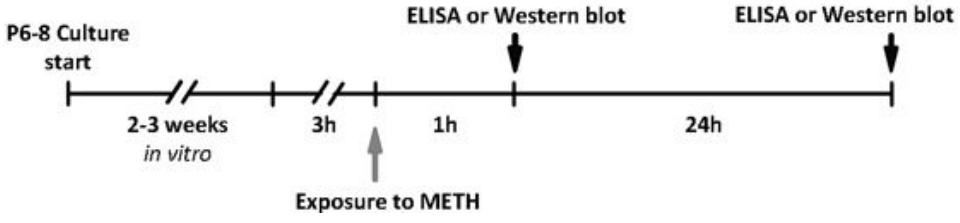


Figure 4.2. *Scheme of the experimental protocol to evaluate the extracellular and intracellular levels of TNF-α, as well as TNFR1 protein levels under conditions of METH-induced toxicity on (A) N9 microglial cell line and (B) mouse hippocampal organotypic slice cultures. For details, see text.*

4.3.5. Cell proliferation assay

Cell proliferation was evaluated by 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) incorporation based on a previous work (Baptista *et al.*, 2012). Microglial cells were treated with METH (0.001-1 mM) and/or 25 µM Z-VAD for 24h, and 10 µM BrdU was added in the last 2h of the treatment session. Cells were then fixed in 4% PFA for 30 min and rinsed in 0.01 M PBS. BrdU was unmasked with 1% Triton X-100 for 30 min, ice-cold 0.1 M HCl for 20 min, and 2 M HCl for 40 min at 37°C, following neutralization in sodium borate buffer (0.1 M $Na_2B_4O_7.10H_2O$, pH 8.5; Sigma-Aldrich) for 15 min at RT and incubation in a blocking solution with 3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.3% Triton X-100 in 0.01 M PBS for 30 min at RT. Afterwards, microglial cells were incubated with rat anti-BrdU antibody (1:50, AbD Serotec) in 0.01 M PBS containing 0.3% Triton X-100 and 0.3% BSA, ON at 4°C, and then with anti-rat Alexa Fluor 488 antibody for 1h at RT. Nuclei were then counterstained with Hoechst 33342, cell preparations were mounted using Dako fluorescent medium and fluorescent images were recorded using Axiovert 200M fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

4.3.6. Immunocytochemistry

Microglial cells were exposure to 1 mM METH during 24h and then rinsed with 0.01 M PBS, fixed with 4% PFA during 30 min at RT, permeabilized with

acetone for 3 min at -20ºC and blocked with 0.01 M PBS containing 10% FBS for 1h at RT. Afterwards, cells were incubated with goat polyclonal antibody ionized calcium binding adaptor molecule-1 (Iba-1; Abcam, Cambridge, MA, USA) ON at 4ºC, that is a specific marker for microglia (Ahmed *et al.*, 2007). After rinses, microglial cells were incubated with anti-goat Alexa Fluor 488 (1:200; Invitrogen, Inchinnan Business Park, UK) together with Rhodamine Phalloidin (1:200, Molecular Probes, Invitrogen), in order to visualized the actin filaments, for 1h 30 min at RT. Finally, cultures were mounted in Dako fluorescence medium (Dako, Glostrup, Denmark) and images were captured using a LSM 710 Meta confocal microscope (Carl Zeiss, Göttingen, Germany).

4.3.7. Western blot analysis

After exposure to METH for 1h or 24h, microglial cells (Fig. 4.2A) and hippocampal slices (Fig. 4.2B) were lysed on ice in RIPA buffer [0.15 M NaCl, 0.05 M Tris-base, 0.005 M ethylene glycol tetraacetic acid (EGTA), 0.5% sodium desoxicolate (DAC), 0.1% sodium dodecyl sulfate–polyacrylamide (SDS) and 1% X-Triton, pH 7.5] supplemented with protease inhibitor cocktail tablets (Roche Applied Sciences) and centrifuged at 17968 x*g* for 20 min. Supernatants were quantified using the BCA method and stored at -20°C until further use. Total proteins (40 µg or 50 µg for N9 microglial cell line or mouse hippocampal organotypic slice cultures studies, respectively) were separated by electrophoresis on SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidine difluoride membrane (PVDF; Milipore, Madrid, Spain), and then blocked with 5% non-fat milk for 1h at RT. Following incubation ON at 4ºC with rabbit anti-TNFR1 primary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, USA), the membranes were washed. Afterwards, the membrane was incubated for 1h at RT with anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:20000; Amersham, GE Healthcare Life Science, USA) and visualized using ECF reagent (Amersham) on Typhoon FLA 9000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Immunoblots were reprobed with β-actin antibody (1:10000, Sigma-Aldrich) or mouse monoclonal glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) antibody (1:1000; Abcam, Cambridge, UK) to ensure equal sample loading, and densitometric analyses were performed using the ImageQuant version 5.0 software.

4.3.8. Statistical analysis

The results are expressed as mean ± SEM. Data were analyzed using one tailed Mann-Whitney test for comparison between two groups, or multiple level analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post hoc test, as indicated in figure legends. Data were considered to be statistically different at $p < 0.05$. For the quantification of TUNEL and BrdU positive cells, six independent microscopy fields *per* coverslip with 20x and 40x objective were acquired, respectively, and results are expressed as percentage of total cells stained with Hoechst 33342 *per* each field (n = microscopy field). In studies involving hippocampal slice cultures, the value of PI uptake induced by 0.5 mM METH was set to 100%. Moreover, the independent samples (n) were defined to be the number of independent hippocampal slices. For western blot and ELISA assay "n" corresponds to the number of independent experiments.

4.4. Results

4.4.1. Effect of METH on microglia: cell death, morphological changes and proliferation

It has been previously suggested that microglia activation contributes to METH related neuropathology (LaVoie *et al.,* 2004), but there is no evidence concerning the direct effect of this drug on microglial cells. Thus, in the present study we aimed to clarify if METH induces microglial cell death. For that purpose, we exposed N9 microglial cells to different concentrations of METH (0.1 - 4 mM) during 24h, and apoptotic cell death was evaluated by TUNEL assay. We observed that METH significantly increases the number of apoptotic cells in a concentration-dependent manner (Figure 4.3A and B). Specifically, at a lower concentration there were no significant differences comparing to control

(control: 0.90 ± 0.12%, n=49; 0.1 mM METH: 1.06 ± 0.28% of total cells, n=10; Figure 4.3A). However, at METH concentrations above 0.5 mM there was a significant increase of cell death as follows: 0.5 mM - 12.63 ± 1.21% of total cells, n=10; 1 mM - 36.44 ± 2.31%, n=37; 2 mM - 69.12 ± 2.53%, n=10; 4 mM - 85.75 ± 1.84% of total cells, n=10 (Figure 4.3A and B). Based on these results we chose to use 1 mM METH (EC_{50}) in the subsequent studies. Moreover, in order to confirm that METH triggers microglial apoptosis, cells were co-exposed to 1 mM METH plus Z-VAD (25 μM), a cell-permeant pan caspase inhibitor. We concluded that Z-VAD alone did not have any effect (2.14 ± 0.42% of total cells, n=20), but was able to completely prevent cell death induced by 1 mM METH (3.8 ± 0.53% of total cells, n=20; Fig. 4.3A).

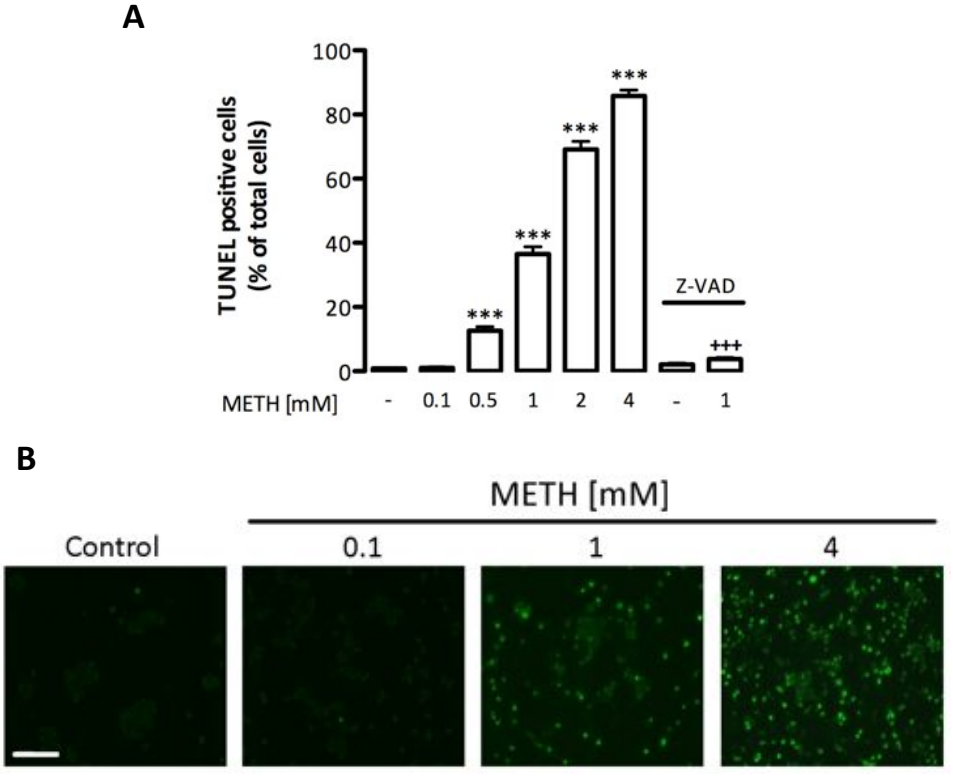


Figure 4.3. *METH induces microglial cell death. (A) METH increases the number of TUNEL-positive cells in a concentration-dependent manner (0.1-4 mM for 24h). The treatment with Z-VAD (25 μM) completely prevented the apoptotic cell death induced by 1 mM METH. The results are expressed as percentage of total cells ± SEM (n=10-25).* $^{***}p < 0.001$*, Dunnett's*

multiple comparison test, significantly different when compared to control. $^{+++}p < 0.001$ *- Bonferroni's multiple comparison test, significantly different comparing with 1 mM METH. (B) Representative fluorescence images of TUNEL positive cells following treatment with 0.1, 1 or 4 mM METH. Scale bar, 20* μm.

In order to evaluate possible morphological changes in survival cells, we performed Iba-1 and F-actin staining (Figure 4.4). Untreated N9 microglial cultures showed considerable ramifications, particularly lamellipodium- and filopodium-like structures, indicating a surveillance state. However, METH led to an arrangement of the actin cytoskeleton and cells acquired a round shape with retracted filopodia characteristic of microglial activation (Figure 4.4). In addition to these morphological alterations we also observed an increase of Iba-1 immunoreactivity induced by METH (Figure 4.4).

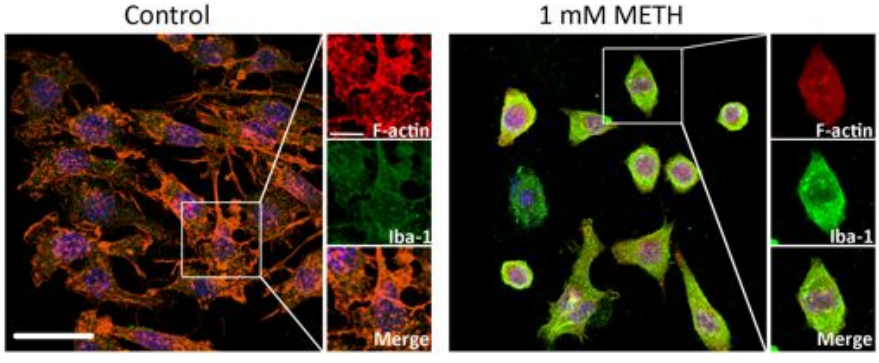


Figure 4.4. *METH modulates microglial activation by change their morphology. Representative confocal images of F-actin (red) and Iba-1 (green) immunoreactivity in N9 microglial cells under (A) control conditions and (B) exposed to 1 mM METH. Cells were also staining with Hoechst 33342 (blue). Scale bar, 20 and 50* μm.

The effect of METH on microglia proliferation was also studied. We found that low concentrations of METH (0.001 and 0.01 mM) stimulated microglia proliferation (control: 49.45±2.65%, n=33; 0.001 mM METH: 71.10±1,80%, n=23; 0.01 mM METH: 74.27±1,83% of total cells, n=24; Figure 4.5). On the other hand, higher concentrations of METH (0.1 and 1 mM) decreased cell proliferation to 16.6±1,2% (n=21) and 13.15±0.90% of total cells (n=35), respectively (Figure 4.5). It is noteworthy that 0.1 mM METH did not induce cell

death, which demonstrates that METH has also a negative impact on microglial proliferation. Moreover, since 1 mM METH induced cell death we further co-exposed the cells with Z-VAD to clarify if the decreased in the number of BrdU positive cells was due to cell death instead of a direct effect on proliferation. Interestingly, Z-VAD reduced, but not completely, the effect of METH (35.45±3% of total cells, n=10, Figure 3) showing that this toxic concentration negatively affects both cell viability and proliferation.

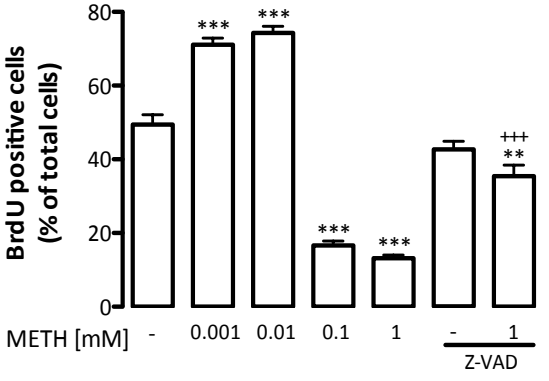


Figure 4.5. *METH has a dose-dependent effect in N9 microglial cell line proliferation. METH treatment (24h) induces increase of BrdU-positive cells at 0.001 and 0.01 mM while leads to a decrease at 0.1 and 1 mM. The co-incubation with Z-VAD (25 mM) was not able to completly prevent 1 mM METH-induced decrease in the number of BrdU positive cells. The results are expressed as percentage of total cells ± SEM (n=10-33).* $^{**}p < 0.01$, $^{***}p < 0.001$ *- Dunnett's multiple comparison test, significantly different when compared to control.* $^{+++}p < 0.001$ *- Bonferroni's multiple comparison test, significantly different comparing with 1 mM METH.*

4.4.2. METH-induced alterations on microglial TNF-α

Microglia activation by inflammatory stimuli increases the synthesis and release of pro-inflammatory cytokines such as TNF-α (Hanisch, 2002). To evaluate the possible changes in microglial TNF system triggered by METH, we measured the release and intracellular levels of this cytokine by ELISA at 1h and 24h after drug exposure. We observed that following 1h of METH exposure, both extracellular (Figure 4.6A) and intracellular (Figure 4.6B) levels were significantly increased to 1265 ± 244.4 pg/ml (n=5) and 2692.0 ± 287.3 pg/mg of total protein (n=7) respectively, when comparing with control (132.2 ± 43.1

pg/ml, n=8 and 1032 ± 214.3 pg/mg of total protein, n=7, respectively; Figure 4.4A and B). Moreover, after 24h there were no significant changes in the released levels (124.8 ± 12.36 pg/ml, n=3) when comparing to control (76.95 ± 1.76 pg/ml, n=3; Fig. 4.6C), but there was an increase of the intracellular levels (control: 473.60 ± 12.59, n=3; 1 mM METH: 946.4 ± 68.89 pg/mg total protein, n=3; Figure 4.6D).

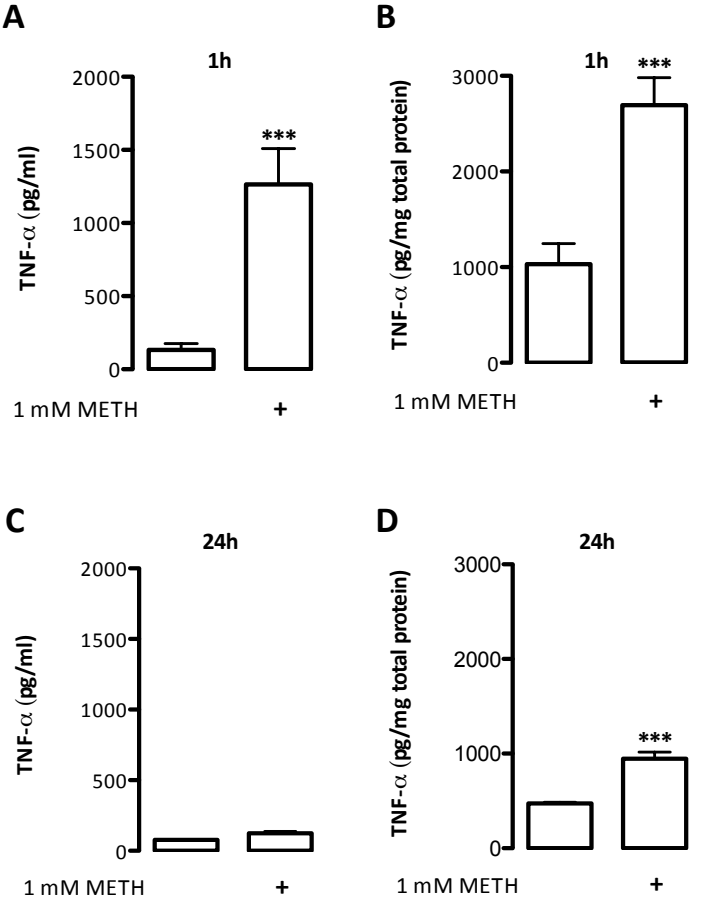


Figure 4.6. *METH triggers an early increase of TNF-α release and production. The effect of 1 mM METH on the (A, C) extracellular d and (B, D) intracellular levels of TNF-α was evaluated after (A, B) 1h and (C, D) 24h of drug exposure by ELISA. Data are expressed as mean ± SEM of pg/ml for extracellular and pg/mg total protein for production (n=3-13) levels.* $^{***}p < 0.001$ *- Mann-Whitney post-test, significantly different from control.*

Since METH induced significant alterations in TNF-α levels, we further investigated if this drug also interferes with the expression of TNFR1. By western blot analysis, we could observe a significant increase in TNFR1 protein levels at 1h post-METH (1 mM) to 164.10 ± 13.04% of control (n=6; Figure 4.7), but no differences were verified after 24h (data not shown).

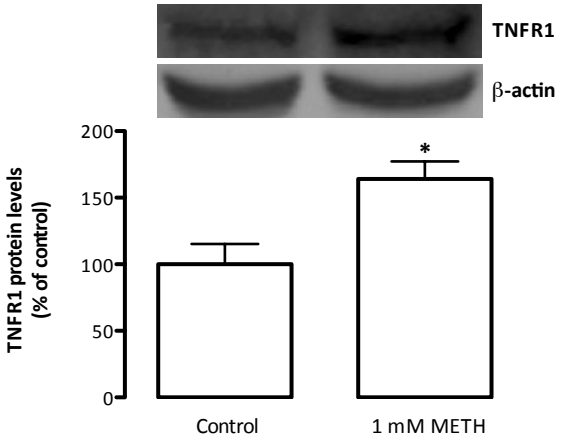


Figure 4.7. *METH increases TNFR1 protein levels. Quantification of TNFR1 in N9 microglial cells at 1h post-METH exposure. Above the bars, representative western blot images of TNFR1 (55 kDa) and β-actin (42 kDa) are shown. The results are expressed as mean % of control ± SEM (n=3-6).* $^{*}p < 0.05$ *- Mann-Whitney posttest, significantly different from control.*

4.4.3. Protective effect of TNF-α against METH-induced microglial cell death

The role of cytokines in response to brain injury remains unclear, in part due to its dual effect (Quintanilla *et al.,* 2004, Bernardino *et al.,* 2005b) that generally depends on the environment, concentration and stimuli duration (Deverman & Patterson, 2009). Thus, since we have shown that METH increases TNF-α release, our next approach was to investigate the role of the endogenous cytokine under METH-induced microglial cell death. For that, we quantified the number of TUNEL positive cells in the presence of TNF-α neutralizing antibody (Figure 4.8A) and observed that 100 μg/ml TNF-α antibody was not able to prevent cell death induced by 1 mM METH (control: 0.5 ± 0.09%, n=84; METH: 28.3 ± 1.41%, n=86; METH+TNF-α antibody: 24.0 ± 2.05% of total cells, n=23;

Figure 4.8A). Moreover, TNF-α antibody *per se* did not induce microglial toxicity (2.5 ± 0.68% of total cells, n=13; Figure 4.8A). Afterwards, we aimed to clarify the effect of exogenous TNF-α on METH-induced microglial cell death. The concentration of 1 ng/ml TNF-α was chosen based on previous studies (Bernardino *et al.,* 2005), and it was not toxic to N9 cell line (control: 0.53 ± 0.09%, n=17; 1 ng/ml TNF-α: 0.72 ± 0.14%, n=17, Figure 4.8B). Indeed, we showed that 1 ng/ml TNF-α completely prevented toxicity induced by 1 mM METH on microglia (METH: 28.28 ± 1.41%, n=86; METH + TNF-α: 2.86 ± 0.46% of total cells, n=25; Figure 4.8B).

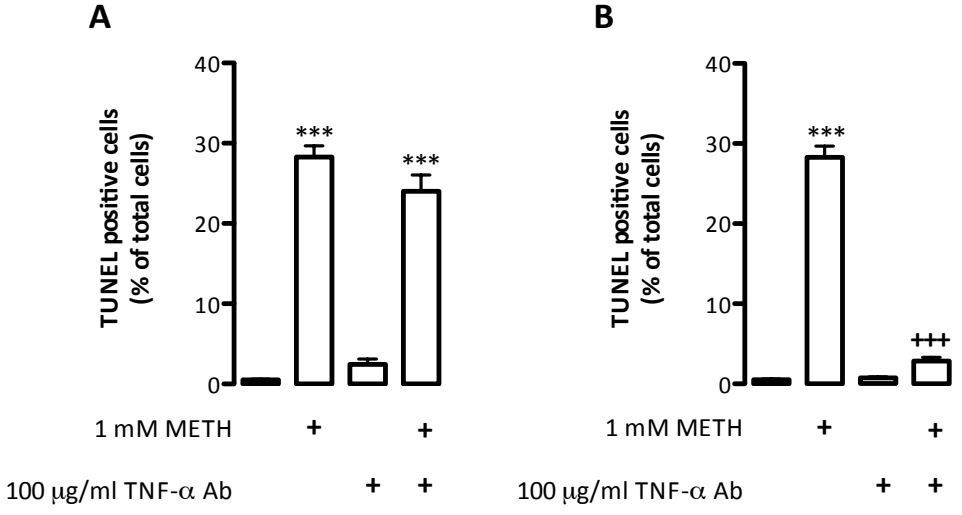


Figure 4.8. *Exogenous TNF-α prevents microglial cell death induced by METH. The increase in the number of TUNEL positive cells induced by 1 mM METH (24h) was not changed by (A) TNF-α antibody but it was completely prevented by (B) TNF-α (1 ng/ml). The results are expressed as mean % of total cells ± SEM (n=17-84).* $^{***}p < 0.001$ *- Dunnet's post-test, significantly different when compared to control.* $^{+++}p < 0.01$ *- Bonferroni's post-test, when compared with 1 mM METH.*

4.4.4. Alterations in TNF system induced by METH on mouse organotypic slice cultures

The results described above clearly show that TNF-α has an importantly protective role against METH toxicity in microglial cells, but we also aimed to evaluate the possible changes in TNF system induced by METH on a more

complex *in vitro* system. For that we used mouse hippocampal organotypic slice cultures, and accordingly to the data obtained in N9 microglial cell line, we verified that at 1h post-METH (0.5 mM) occurred an up-regulation in the extracellular (Figure 4.9A) and intracellular levels of TNF-α (Figure 4.9B) to 334.0 ± 34.9 pg/ml (n=15) and 14800 ± 826.7 pg/mg total protein (n=5), respectively, when compared with control (145.3 ± 6.6 pg/ml, n=14 and 5381.0 ± 1126.0 pg/mg total protein, n=6). However, after 24h, METH did not induce changes neither in the extracellular levels (control: 50,1 ± 12,7 pg/ml, n=3; METH: 34,3 ± 3,2 pg/ml, n=3; Figure 4.9C) nor in the production of TNF-α (control: 2725.0 ± 148.8 pg/mg total protein, n= 3; METH: 2874.0 ± 156.8 pg/mg total protein; Figure 4.9D).

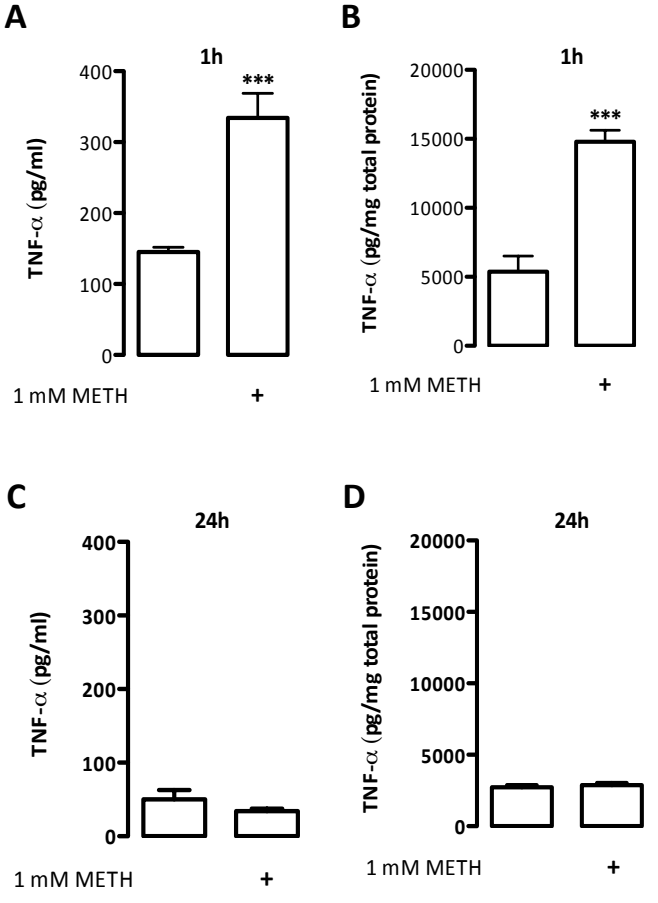


Figure 4.9. *Early up-regulation of intracellular and release TNF-α protein levels induced by METH. The effect of 0.5 mM METH on the (A, C) extracellular and (B, D) intracellular levels*

of TNF-α was evaluated after (A, B) 1h and (C, D) 24h of drug exposure by ELISA. Data are expressed as mean ± SEM of pg/ml for extracellular and pg/mg total protein for intracellular (n=3-15) levels. $^{***}p < 0.001$ - Mann-Whitney post-test, significantly different from control.

Similar to microglial cells, we evaluated the protein levels of TNFR1 following METH treatment and found that in organotypic hippocampal slice cultures the levels of this receptor was increased to 167.5 ± 16.5% (n=11), when compared with non-treated slices (100.0 ± 8.4%, n=8; Figure 4.10).

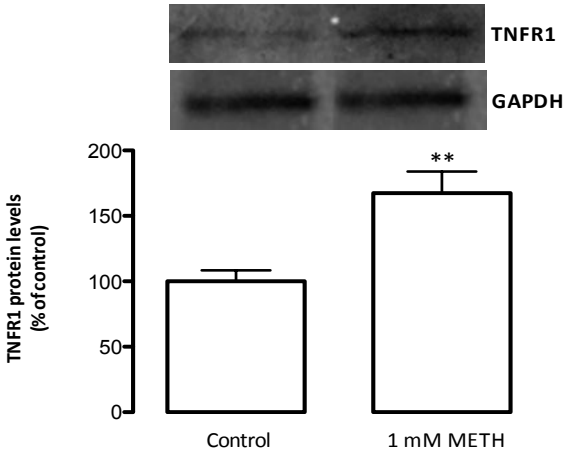


Figure 4.10. *TNFR1 protein levels were up-regulated at 1h post-METH treatment in mouse hippocampal organotypic slice cultures. Above the bars, representative western blot images of TNFR1 (55 kDa) and GAPDH (34 kDa) are shown. The results are expressed as mean % of control ± SEM (n=8-11). $^{**}p < 0.01$ - Mann-Whitney post-test, significantly different from control.*

4.4.5. TNF-α partially protects hippocampal neurons from METH-induced neurodegeneration

It was reported that TNF-α plays a neuroprotective role in METH-induced striatal neurotoxicity (Nakajima *et al.,* 2004). Moreover, our results show that METH induces alterations in the hippocampal TNF system. So, we further aimed to clarify the effect of TNF-α under hippocampal neurodegeneration induced by METH. We verified that TNF-α by itself was not toxic to hippocampal pyramidal neurons (CA3: 29.2 ± 9.8, n=6; CA1: 27.7 ± 5.7, n=6; Figure 4.11) comparing

with control (CA3: 12.3 ± 1.5, n=10; CA1: 13.9 ± 3.0, n=10; Figure 4.11). Furthermore, in this *in vitro* model, the pre-treatment with exogenous TNF-α was able to protect the CA3 (52.1 ± 8.7, n=9; Figure 4.11A e C) and CA1 (37.8 ± 8.8, n=9; Figure 4.11B e C) hippocampal sub-regions, when compared with METH treatment *per se* (CA3: 100.0 ± 6.2, n=8; CA1: 100.0 ± 4.5, n=8). However, in contrast with the results obtained in microglial cells, the protective effect of TNF-α in organotypic cultures was only partial.

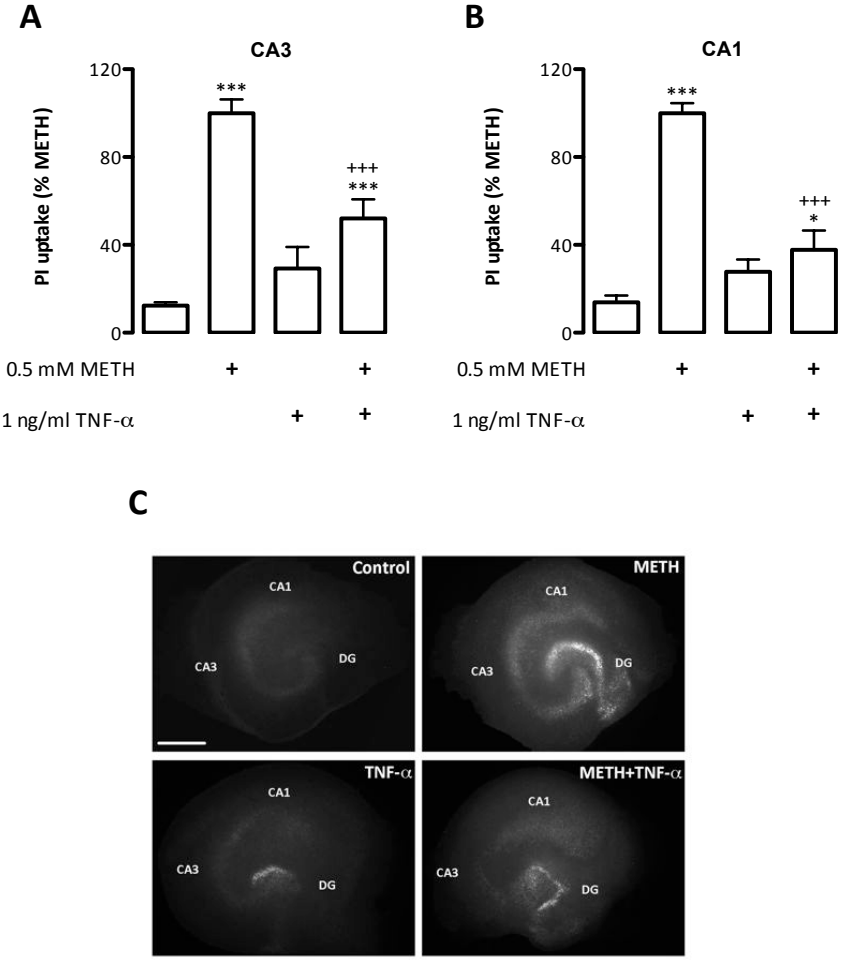


Figure 4.11. *Exogenous TNF-α protects hippocampal cultures from METH neurodegeneration. The pre-treatment with exogenous TNF-α (1 ng/ml) partial prevents the neurotoxicity induced by 0.5 mM METH in (A) CA3 and (B) CA1 pyramidal cell layers. The PI uptake induced by 0.5 mM METH was set to 100%. Data are shown as mean value ± SEM, n=10-6.* $^{*}p < 0.05$; $^{***}p < 0.001$ *- Dunnett's post-test, significantly different from control (no*

drug exposure). $^{+++}p < 0.001$ - Bonferroni's post-test, statistical significance when compared with 0.5 mM METH. (C) Representative fluorescence microscopy images of PI uptake in hippocampal organotypic slice cultures under several experimental conditions. Scale bars: 500 µm

4.5. Discussion

It has been extensively described that METH triggers neuronal dysfunction or/and death (Deng *et al.*, 2001, 2002; Cadet *et al.*, 2005; Gonçalves *et al.*, 2010) and also activation of glial cells, namely microglia (LaVoie *et al.*, 2004; Sekine *et al.*, 2008; Gonçalves *et al.*, 2010), with a consequent increase of pro-inflammatory cytokines production. However, the role played by these cytokines under conditions of METH-induced microglial toxicity is poorly understood. In the present study, we report that METH induces microglial cell death as well as hippocampal neurodegeneration, which is accompanied by an early increased in the extracellular and intracellular levels of TNF-α. However, we demonstrate that the up-regulation of this pro-inflammatory cytokine does not contribute to METH-induced cell death. Yet, exogenous TNF-α has a protective effect against microglia and neuronal toxicity induced by METH.

The involvement of inflammatory events, such as gliosis (Lavoie *et al.,* 2004; Thomas *et al.,* 2004; Sriram *et al.,* 2006a; Gonçalves *et al.,* 2010) and pro-inflammatory cytokines release/production (Sriram *et al.,* 2006a; Gonçalves *et al.,* 2010, 2008; Tocharus 2010) has been recently suggested as playing an important role in METH-induced brain dysfunction. Our group showed that a single high dose of METH (30 mg/kg; i.p.) led to a rapid up-regulation of TNF-α mRNA levels in the mouse hippocampus and frontal cortex (Gonçalves *et al.,* 2008). Moreover, we also demonstrated that the same METH treatment triggered a neuroinflammatory response characterized by microglia activation and astrogliosis, as well as by changes in TNF system protein levels, namely an early and late up-regulation of TNFR1 and TNF-α protein levels, respectively (Gonçalves *et al.,* 2010). Concerning *in vitro* studies, there is only one report

that has approached this issue by demonstrating that, in HAPI microglial cells, a non-toxic concentration of METH (0.8 mM, 6h exposure) increased the mRNA levels of different pro-inflammatory cytokines, including TNF-α, followed by production of reactive oxygen/nitrogen species (Tocharus *et al.,* 2010). Importantly, in our model, we detected that METH-induced toxicity was accompanied by morphological changes in microglial cells, from a resting to a full-activated state. Furthermore, microglia not only changed their morphology and cell surface receptor expression but also its number (Streit, 2000). Here, we observed that METH affects microglial proliferation at a concentration-dependent manner with lower concentrations causing an increase in the cell number, whereas higher concentrations decreased cell proliferation. Our data also demonstrated that METH toxicity caused the increase of extracellular and intracellular levels of TNF-α together with up-regulation of protein levels of TNFR1, which demonstrated that this drug significantly interferes with this pro-inflammatory cytokine signaling. In concordance with our previous work (Gonçalves *et al.,* 2008), we found that these alterations occurred very quickly since METH evoked the release of TNF-α just after 1h. Interestingly, we verified that the blockade of endogenous TNF-α did not affect microglial cell death induced by METH, which suggest that the up-regulation of this molecule release is a consequence of METH toxicity and not a cause. On the other hand, the application of a low concentration of exogenous TNF-α completely prevented the apoptotic microglial cell death induced by the drug. In fact, Nakajima *et al.* (2004) reported an up-regulation of rat striatal TNF-α levels following a repeated treatment with METH (2 mg/kg for 5 days, s.c.), which was associated with a neuroprotective effect. Specifically, they showed that exogenous TNF-α (4 μg; i.c.v.) blocked locomotor-stimulating and rewarding effects of METH (4 mg/kg; four times at 2-h intervals), and also decreased the extracellular levels of striatal dopamine and potentiated its uptake into synaptossomes (Nakajima *et al.,* 2004). Furthermore, Niwa *et al.* (2007) showed that TNF-α protects against METH-induced rewarding effects and sensitization by regulating extracellular DA levels in mice nucleus accumbens.

It is well known that METH affect different brain cells, and neurons seems to be highly sensitive to this drug. Indeed, our group demonstrated that hippocampal neurons display a greater susceptibility to METH (Gonçalves *et al.,* 2010; Martins *et al.,* 2011). Accordingly, the present study shows that METH increased extracellular and intracellular TNF-α levels, and also the TNFR1 protein levels in hippocampal slices. Despite the fact that TNFR1 is normally associated with apoptosis because it contains a cytoplasmatic death domain (Tartaglia *et al.,* 1993b), several authors have been demonstrated that TNFR1 signaling pathway can initiates pro-survival cascades and cellular proliferation (see review McCoy & Tansey, 2008). Furthermore, we observed that TNF-α pre-treatment partial prevented METH-induced hippocampal neurotoxicity, namely in CA3 and CA1 pyramidal cell layers, which is in accordance with the results obtained by Bernardino et al., (2005) showing a protective effect of TNF-α against AMPA-mediated excitotoxicity. Also, this pro-inflammatory cytokine showed to be a potent neuronal protector in β-amyloid-treated rat hippocampal neuronal cultures (Orellana *et al.*, 2007). Moreover, Rodriguez and collaborators (2009) demonstrated that depletion of TNF-α delays healing of the hippocampus following a virus-induced encephalitis. In addition, the same authors observed that after intracranial injection of TNF-α, the hippocampus quickly recovers from the injury and this reparative function was mediated by TNFR1 activation (Rodriguez *et al.*, 2009). In contrast to microglia, the neuroprotection in hippocampal slices was only partial, which may be explained by the fact that organotypic hippocampal slice cultures are a complex system that consists of several cells such as neurons, microglia, astrocytes and oligodendrocytes (Lossi *et al.*, 2009), which creates a cellular dynamic and interplay very different from that observed in the cell line. In fact, in these cultures we did not evaluate the cellular source of TNF-α.

Our results highlight the toxic effect of METH on microglial cells since it induced cell death together with an increase in the release and production of TNF-α and up-regulation of TNFR1 protein levels. These features are maintained in hippocampal slice cultures following METH insult. Interestingly, endogenous

cytokines *per se* did not affect METH-induced cell death, which suggest that alterations on this system are a consequence rather than a cause of METH induced microglial apoptosis. Moreover, exogenous low concentrations of TNF-α provided a protective effect against METH-induced microglia and neuronal toxicity. Thus, our data suggest that the activation of TNF signaling is an important target to prevent or at least to minimize the toxic effects of METH.

Chapter 5

Methamphetamine-induced changes in the mice hippocampal neuropeptide Y system: implications for memory impairment

5.1. Abstract

Methamphetamine (METH) is a psychostimulant drug that causes irreversible damage to the brain leading to several neurological and psychiatric abnormalities, including cognitive deficits. Neuropeptide Y (NPY) is abundant and has important functions in the mammalian central nervous system (CNS), including modulation of cognition. Previously, it was demonstrated that METH induces significant alteration in striatal NPY, Y_1 and Y_2 receptor mRNA levels of mouse. However, the impact of this drug on the hippocampal NPY system and its consequences remain unknown. Thus, in the present study we investigated the effect of METH intoxication on mouse hippocampal NPY levels, functionality of its receptors, and on memory performance. Results show that METH increased NPY, Y_2 and Y_5 receptor mRNA levels, as well as total NPY binding that was accompanied by up- and down-regulation of Y_2 and Y_1 functional binding, respectively. Moreover, METH impaired memory performance and AKT/mTOR pathway that could both be prevented by the Y_2 receptor antagonist, BIIE0246. These findings demonstrate that METH interferes with the hippocampal NPY system, which seems to be associated with memory impairment. Specifically, we concluded that Y2 receptors are involved in memory deficits induced by METH intoxication.

5.2. Introduction

Methamphetamine (METH) is a highly addictive psychostimulant, leading to neurological and psychiatric abnormalities. Indeed, METH abusers show structural brain abnormalities, specifically in the hippocampus and prefrontal cortex, and are associated with cognitive deficits (Simon *et al.*, 2000; Thompson *et al.*, 2004; Salo *et al.*, 2009). Similarly, METH leads to impairment in animal spatial, short- and long-term recognition and perceptual memories (Cherng *et al.*, 2007; O'Dell *et al*., 2011; Lee *et al*., 2011). In accordance with these observations, we previously showed that an acute METH administration alters the expression of several rat hippocampal ionotropic glutamate receptor subunits, which seems to be correlated with the hippocampal-dependent memory impairment observed (Simões *et al.*, 2007). It is known that METH induces neuropathology *via* several mechanisms, including monoaminergic system damage, excitotoxicity and neuroinflammation (Silva *et al.*, 2010). In fact, METH intoxication triggers hippocampal gliosis and cytokine production (Gonçalves *et al.*, 2008; Gonçalves *et al.*, 2010), together with significant cytoskeleton, synaptic and axonal protein alterations, but without evidence of cell death (Gonçalves *et al.*, 2010).

NPY is a neuromodulator widely distributed in the hippocampus (Gehlert, 2004) and acts *via* Y_1, Y_2 and Y_5 receptor subtypes (Silva *et al*. 2005b; Xapelli *et al*. 2006). This peptide has several important functions such as the regulation of appetite and circadian rhythm (Berglund *et al*. 2003), cognitive processing (Thorsell *et al*. 2000; Karl *et al*. 2008; Sørensen *et al*. 2008) and neuroprotection (Silva *et al*. 2005a). Moreover, seizures up-regulate hippocampal NPY levels and it has been considered to be an endogenous antiepileptic agent (Woldbye *et al.*, 1996; Silva et al, 2003b, 2005b; Xapelli et al, 2007). However, NPY-increased levels could also affect other hippocampal functions, including learning and memory. In fact, some authors have clearly demonstrated that hippocampal NPY overexpression was accompanied by hippocampal activity-dependent

plasticity reduction in excitatory synapses, which was associated with acquisition and retention deficits in spatial memory (Thorsell *et al.*, 2000; Sørensen *et al.*, 2008a). Furthermore, *in vitro* studies showed that hippocampal NPY reduces calcium influx and glutamatergic transmission mainly via presynaptic Y_2 receptors (Silva *et al.*, 2003a), and these effects resulted in the suppression of long-term potentiation (LTP) (Qian *et al.*, 1997). Regarding the role of NPY and its receptors under METH consumption, limited data are available. It is known that multiple high doses of METH produced an increase of rat striatal pre-pro-NPY mRNA-expressing neurons (Horner *et al.*, 2006). Similarly, Thiriet and collaborators (2005) demonstrated an up-regulation of striatal NPY mRNA, together with a down-regulation or biphasic changes in Y_1 or Y_2 receptor expression, respectively. The authors also stated that NPY protects against METH-induced striatal neurotoxicity through Y_1 and Y_2 receptors (Thiriet *et al.*, 2005). Nevertheless, the role of the hippocampal NPY system under METH consumption has never been addressed before.

Thus, the aim of the present study was to investigate the possible changes in the mouse hippocampal NPY expression, as well as its receptors expression and functionality triggered by METH intoxication at several time-points post-administration. Moreover, memory performance and the signaling pathway underlying such alterations were also evaluated. We concluded that METH increases hippocampal NPY levels and differently affects the levels and functionality of NPY receptors. Further, we suggested that the increase of Y_2 receptors activation could be implicated in METH-induced memory impairment. Overall, our data provide a potential new role for the hippocampal NPY system, particularly the Y_2 receptor, under METH intoxication by demonstrating how it may compromise memory.

5.3. Materials and Methods

5.3.1. Animal procedure and drug treatments

Three-month-old male C57BL/6J mice (20-30 g body weight; Charles Rivers Laboratories) were housed with access to food and water *ad libitum* on a 12h light/dark cycle in a temperature-controlled room. Behavioral experiments were performed in a sound-attenuated room, where mice were habituated for 1h. Experimental procedures for the use of animals in the laboratory were performed accordingly to the guidelines of the European Community (86/609/EEC), the Portuguese law (DL 129/92) and the Animal Experimentation Inspectorate, Denmark. The authors attest that all efforts were made to minimize animal suffering and the number of animals used.

Animals were anesthetized with Avertin® [0.013 ml/g body weight; intraperitoneal (i.p.)]. Afterwards, a stainless-steel guide cannula (Plastic One) was implanted unilaterally above the right lateral ventricle, according to the coordinates described by Decressac et al (2010), and was fixed to the skull with dental cement. Mice were individually housed for at least 3 days, followed by drug treatments using an injector cannula (Plastics One). Dummy cannulas (Plastics One) were used during experiments, except when injection was conducted.

METH [(+)-methamphetamine hydrochloride, Sigma-Aldrich; 30 mg/kg body weight; i.p.] was administered and mice were sacrificed after different time-points as follows: 1h, 24h, 3 and 7 days post-injection. Importantly, METH intoxication protocol selected in the present study has been successfully used by us and others (Zhu *et al.*, 2006; Gonçalves *et al.*, 2010; Martins *et al.*, 2011; Tulloch *et al.*, 2011), and did not cause seizures in treated-animals. Y_2 receptor antagonist was dissolved in 1% DMSO/saline solution and injected intracerebroventricularly (i.c.v., 2 µl of 1.0 nmol BIIE0246; Tocris Bioscience) as previously described (Redrobe *et al.*, 2002; Thorsell *et al.*, 2002), and 45 min before METH treatment. Control animals received 1% DMSO/saline (i.c.v.).

5.3.2. Brain sectioning for *in situ* hybridization and binding studies

Mice were sacrificed post-METH treatment and their brains were removed, frozen on dry ice, and stored at -80ºC. Coronal sections (12 µm) were cut on a cryostat (Thermo Shandon Inc.) across the hippocampus, thaw-mounted onto slides and stored at -80ºC until used.

5.3.3. *In situ* hybridization studies

One glass slide from each animal was defrosted for 30 min at room temperature (RT) to evaporate condensed water. Next, the slides were fixed for 5 min in 4% paraformaldehyde (PFA) solution, and placed for 5 min in 0.01 M phosphate buffered saline (PBS). Afterwards, the slides were placed in 70% ethanol for 5 min and stored in 95% ethanol at 4ºC until hybridization. Synthetic DNA complementary oligonucleotide probes (DNA Technology A/S, Aarhus, DK) were used for evaluation of NPY and Y_1, Y_2 and Y_5 receptor mRNA levels (Table 5.1; Woldbye *et al.*, 2005, 2010). Further, to enhance sensitivity, an equal molar mixture of two non-overlapping probes was used for each NPY receptor.

Table 5.1. List of the sequence of the probes used for evaluation of NPY and Y_1, Y_2 and Y_5 receptor mRNA

	Sequence of the probe
NPY	5'-GTC-CTC-TGC-TGG- CGC-GTC-CTC-GCC-CGG-ATT-GTC-CGG-CTT-GGA-GGG-GTA-3'
Y_1	5'-GCA-GAC-GGC-GAA-GGC-GAC-CAC-AAT-GGA-GAG-CAG-CAT-GTT-GAT-TCG-CT-3' 5'-GTG-GTT-GCA-GGT-GGC-AAT-GAT-CTG-GTG-GTT-CCA-GTC-GAA-CAC-AGT-GTT-3'
Y_2	5'-GCA-AGA-TGA-TGG-AGC-AGT-AGG-CCA-ATA-TGA-GGA-TCA-CCT-GCA-CCT-CG-3' 5'-GAG- CAA-TGA-CTG-TCA-AAG-TTA-TTG-TGG-ACA-CTT-GTA-CCG-CCA-GAC-CCA-G-3'
Y_5	5'-CGA-GTC-TGT-TTT-CTT-TGT-GGG-ACA-ATC-CAC-AGC-TTA-TAC-TCC-TGC-3' 5'-CAC-GCA-TGC-CGT-CTT-CTT-GCT-GTA-CCT-TCT-TCG-GTG-CTT-TCT-GAT-3'

Each probe was labeled with [α^{35}S]dATP (1250 Ci/mmol, Amersham Biosciences, GE Healthcare UK Ltd, Buckinghamshire, UK) using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany) to give

a specific activity of 300,000 cpm/100 µl to the hybridization buffer which contained 50% formamide (v/v) (Fluka), 4x saline sodium citrate (SSC; 1x SSC = 0.15 M NaCl, 0.015 M Na3C6H5O7, pH 7.0 [Sigma-Aldrich]), 10% (w/v) dextran sulphate (Fluka), and 10 mM dithiotreitol (DTT; Sigma-Aldrich). Sections were then incubated at 42ºC overnight (ON). Subsequently, they were washed for 30 min in 1x SSC at 60ºC, passed through a series of 1 min rinses in 1x SSC, 0.1x SSC, 70% ethanol (EtOH), and 95% EtOH at RT, and finally air-dried. The slides were exposed to ^{35}S-sensitive Kodak BioMax MR films together with ^{14}C-microscales (both Amersham Biosciences) for 4 weeks before being developed in Kodak GBX developer. For semiquantitative analysis of mRNA levels, optical densities (nCi/g) based on calibration curves were obtained from ^{14}C-microscales. The analysis of autoradiographic images was performed using Scion Image computer analysis software (NIH, USA). We measured bilaterally over the dentate gyrus (DG), CA1 and CA3 sub-regions. Right- and left-side values were averaged per section and used to calculate the mean for each animal. Background measurements immediately adjacent to each brain section were subtracted from each measurement before calculations. In control experiments, the specificity of the antisense oligoprobes was confirmed by adding corresponding "non-labeled" antisense probes (competitive controls).

5.3.4. NPY receptor binding

Brain slices from the same animals used for *in situ* hybridization were used as previously described (Christensen *et al.*, 2006; Woldbye *et al.*, 2005, 2010). In detail, slices were defrosted at RT for 30 min and then preincubated for 20 min in binding buffer (pH 7.4), containing 25 mM N-[2-hydroxyethyl]-piperazine-NV-[2-ethansulfonic acid] (HEPES), 2.5 mM CaCl2, 1 mM $MgCl_2$, 0.5 g/L bacitracin, and 0.5 g/L bovine serum albumin (BSA). Afterwards, they were incubated for 1h at RT with binding buffer containing 0.1 nM [^{125}I][Tyr^{36}]mono-iodo-PYY (4000 Ci/mmol; porcine synthetic, IM259; Amersham Biosciences) alone or together with 1 µM NPY (mouse synthetic, H-5375; Bachem AG, Bubendorf, Switzerland) to visualize total binding to all NPY receptor subtypes or nonspecific binding,

respectively. Following a quick rinse, the slices were washed in binding buffer for 2x 30 min at RT and then air-dried ON. Then, they were exposed for 4 days at -20ºC to ^{125}I-sensitive Kodak Biomax MS films with ^{125}I-microscales (both Amersham Biosciences) before developing the films (Kodak GBX developer). Calibration was based on the ^{125}I-microscales, and optical densities were measured as explained above over the DG, CA1 and CA3 sub-regions, using computer-assisted image analysis. Total specific NPY-sensitive binding was calculated by subtracting nonspecific binding from total binding.

5.3.5. NPY-stimulated [^{35}S]GTPγS binding autoradiography

As previously described by us (Christensen *et al.*, 2006; Silva *et al.*, 2007; Woldbye *et al.*, 2010; Gotzsche *et al.*, 2012), brain sections were defrosted and air-dried for 30 min at RT before being rehydrated in assay buffer A [50 mM Tris-HCl, 3 mM MgCl, 0.2 mM ethylene glycol tetra-acetic acid (EGTA), 100 mM NaCl, pH 7.4] for 10 min at RT. Then, the slices were pre-incubated in assay buffer B [assay buffer A + 0.2 mM dithiothreitol (DTT), 1 µM 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Sigma-Aldrich), 0.5% w/v BSA and 2 mM guanosine-5'-diphosphate (GDP; Sigma-Aldrich)] in the presence of NPY receptor antagonists (if applicable) for 20 min at RT to shift all G-proteins into the inactive state. Brain sections were subsequently incubated in assay buffer B + 40 pM [^{35}S]-GTPγS (1250 Ci/mmol; NEG030H250UC; PerkinElmer, DK) for 1h at 25°C with 10^{-6} M NPY (Bachem AG) plus different combinations of NPY receptor antagonists (10^{-6} M each) to visualize contribution of individual NPY receptors: BIBP3226 (Y_1 receptor antagonist; Bachem AG), BIIE0246 (Y_2 receptor antagonist; Tocris Cookson, UK), and L-152,804 (Y_5 receptor antagonist; Tocris Cookson). In each experiment, basal binding was determined by omitting the application of NPY receptor ligands and nonspecific binding by applying 10 µM unlabeled GTPγS (Sigma-Aldrich, DK). In addition, total blocking of NPY-stimulated functional binding was obtained by adding a mixture of BIBP3226, BIIE246 and L-152,804. Then, slices were washed 2x 5min in ice-cold 50 mM Tris-HCl buffer (pH 7.4) followed by a brief rinse in cold deionized water.

Sections were air-dried and exposed to Kodak BioMax MR films (Sigma-Aldrich) for 5 days at -20°C together with ^{14}C standards (Amersham Life Sciences). Functional binding was quantified by measuring optical densities bilaterally over the dorsal CA1 and CA3 (fiber layers), as well as DG (molecular layer) using computer assisted image analysis (Scion Image® analysis program). Right and left values were averaged per section and used to calculate the mean of each animal. Background measurements immediately adjacent to each brain section were subtracted from each measurement before calculations.

5.3.6. Western blot analysis

Mice were sacrificed at 1h or 24h post-METH administration, brains were removed and the hippocampi were dissected on ice. The tissue was then homogenized in lysis buffer (50 mm Tris-HCl pH 7.4, 0.5% Triton X-100, 4°C) supplemented with a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM DTT, 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1 µg/mL antipain and 5 µg/mL pepstatin A; all from Sigma-Aldrich). Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) and protein samples (50 µg) were separated onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), before transferred onto polyvinylidinedifluoride membrane (PVDF; Millipore, Madrid, Spain). After blocking, membranes were incubated ON at 4°C with the following primary antibodies: phospho-Akt-Ser473 (1:1000), phospho-mTOR-Ser2448 (1:1000), phospho-p70S6 kinase-Thr421/Ser424 (1:1000; Cell Signaling Technology, Danvers, MA, USA), and anti-neuropeptide Y (1:1000; Sigma-Aldrich). Afterwards, membranes were incubated with secondary antibodies coupled to horseradish peroxydase (HRP), for 1h at RT, and immunoreactive bands were visualized by enhanced chemifluorescent detection (ECF kit, Amersham Biosciences). The levels of phosphorylation for AKT, mTOR and p70S6 kinase were normalized by reprobing stripped membranes with antibodies raised against the respective total proteins. Besides, the membranes were also reprobed with anti-glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) as a loading control. The immunoblots were analyzed with ImageQuant software to measure the optical density of the bands.

5.3.7. Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (Sigma-Aldrich, St. Louis, MA, USA) at 1h or 24h post-METH administration, and intracardially perfused with 0.01 M PBS followed by 4% PFA in 0.01 M PBS, pH 7.4. Brains were removed and post-fixed ON at 4°C in the same solution, followed by immersion in 20% sucrose in 0.01 M PBS at 4°C for 24h. Afterwards, coronal sections from posterior to anterior hippocampus were cut (30 μm) and collected in 0.01 M PBS until free-floating immunohistochemistry studies. Double-labeling immunofluorescence was performed for NPY and a neuronal marker, neuron-specific class III beta-tubulin (Tuj-1). Briefly, hippocampal sections were rinsed in 0.01 M PBS, blocked with 10% fetal bovine serum (FBS)/0.5% Triton X-100 in 0.01 M PBS for 1h 30 min at RT, and incubated for 24h at 4°C with polyclonal anti-NPY (1:200). Sections were then washed and incubated with Alexa Fluor 488 antibodies (1:200; Invitrogen, Inchinnan Business Park, UK) for 1h 30 min at RT. From this point forward, the slices were protected from light. After washing, we once again blocked the sections with 1% FBS/0.3% Triton X-100 in 0.01 M PBS for 1h 30 min at RT followed by incubation with antibodies against different cell types, ON at 4°C. Neurons were visualized using a mouse anti-Tuj-1 antibody (1:500; Covance, Ermeryville, California, USA). After rinses, slices were incubated for 1h 30 min at RT with Alexa Fluor 594 secondary antibodies (1:200; Invitrogen), and the nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). In addition, immunofluorescence labeling was performed against phospho-AKT and NPY Y_2 receptor. First, hippocampal sections were rinsed in 0.01 M PBS before blocking with 5% goat serum/0.3% Triton X-100 in PBS for phospho-AKT or with 10% FBS/0.5% Triton X-100 in PBS during 1h 30 min at RT, following incubation with primary antibody [anti-phospho-AKT-Ser473 rabbit antibody (1:200; Cell Signaling) and anti-NPY Y_2 receptor (1:100; Alomone Labs, Jerusalem, Israel] for

24h at 4°C. Then, sections were rinsed and hippocampal slices were incubated for 1h 30 min at RT with the secondary antibody Alexa Fluor 488 (1:200; Invitrogen). In the dark, the sections were again washed and stained with Hoechst 33342 (Sigma-Aldrich), finally mounted in Dako fluorescence medium (Dako North America, Carpinteria, USA) and images were recorded using a LSM 710 Meta Confocal microscope (Carl Zeiss, Oberkochen, Germany).

5.3.8. Y-maze test

Spatial working memory was accessed using Y-maze apparatus with three identical black horizontal arms (5 cm wide x 36 cm long x 16 cm high; Panlab) symmetrically disposed at 120° to each other. In the first trial, 5 min after METH or saline injection, mice were placed in the end of a random-assigned arm (start arm) and allowed to explore start and familiar arms, with the third arm (novel arm) blocked, for 8 min. The second trial, 24h post-METH or -saline treatment, each mouse was placed again in the maze with free access to all three arms during 8 min. The exploration time and the percentage number of entries into the novel arm were recorded. The total number of arm entries was measured as an index of locomotor activity to rule out the possible interference of changes in motility with the parameters of learning and memory. The recognition of the novel arm from the other two familiar arms is considered as a memory improvement effect (Cherng *et al.*, 2007).

5.3.9. Novel object recognition test

The novel object recognition task requires that the mice recall which of two objects they have previously been exposed to (Bura *et al.*, 2007). This test took place in an open-field box (30 cm wide x 30 cm long x 58 cm high) with white vertical walls and white floor divided into 9 equal squares (Bura *et al*., 2007). The field was dimly illuminated and the objects to be discriminated were a plastic animal figure (4 cm long x 5.5 cm high) and a Lego® toy (3 cm long x 6 cm high). Before the start of the experiments, mice were individually habituated to the open field for 40 min. The next day, 5 min following METH

administration, animals were submitted to a 10 min acquisition trial (training phase) during which they were placed in the box in the presence of the object A. We recorded locomotor activity (number of squares crossed), rearings and latency to explore object A (animal's snout directed toward the object at a distance ≤ 1 cm). After 24h, a 10 min retention trial (test phase) was performed in order to assess recognition memory. During the test, objects A and B were placed in the open field and locomotor activity, rearings and latency to explore object A (t_A) and object B (t_B) were recorded. A recognition index was defined as ($t_B/(t_A+t_B)$)x100. Objects A and B were counterbalanced so that half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other half first explored object B and then object A. Importantly, no mice showed preference for the different objects (data not shown). To avoid the presence of olfactory traces, the open-field box and the objects were cleaned between tests. The novel object recognition test was conducted 24h after METH treatment in order to evaluate long-term memory (O'Dell *et al.*, 2011; Reichel *et al.*, 2011).

5.3.10. Statistical analysis

The measurements of *in situ* hybridization and receptor binding studies were performed blind to treatments. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test, except for the Y-maze tests that were analyzed using the Mann-Whitney test. Data were considered statistically significant when $p < 0.05$.

5.4. Results

5.4.1. METH increases hippocampal NPY, Y_2 and Y_5 receptor levels

NPY system seems to be altered under several pathological conditions (see review Silva *et al.*, 2005a) but limited information is available regarding this issue (Goodman and Slovitter 1993; Cannizzarro *et al.*, 2003; Thiriet *et al.*,

2005). Nervertheless, Thiriet *et al.* (2005) showed that multiple high doses of METH increases striatal NPY mRNA and induced changes in Y_1 and Y_2 receptor mRNA levels. Thus, we hypothesized that METH intoxication (30 mg/kg; i.p.) could also trigger significant alterations in the hippocampal NPY system. We found that NPY mRNA were up-regulated at 1h post-METH administration (163.3 ± 10.5% of control, n=9; $p = 0.0007$; Figure 5.1A) in the DG cells, whereas in the CA3 sub-field this increase was only significant after 24h (175.9 ± 22.7% of control, n=7; $p = 0.0099$; Figure 5.1A). In the CA1 sub-region, we observed an increase of NPY mRNA levels at both 1h (190.1 ± 15.8% of control, n=6) and 24h post-injection (187.9 ± 22.9% of control, n=7; $p < 0.0001$; Figure 5.1A). Importantly, the NPY mRNA in hippocampal formation returned to basal values 3d post-METH treatment (Figure 5.1A). In figure 5.1B, representative *in situ* hybridization autoradiograms show the increase of NPY mRNA expression in the mouse hippocampus post-1h or 24h METH exposure. Additionally, we showed that METH-induced increases in NPY protein levels at 1h (133.3 ± 3.8% of control, n=9) and even more so at 24h post-injection (166.9 ± 13.3% of control, n=9; $p < 0.0001$; Figure 5.1C). These results were confirmed by immunohistochemical analysis (Figure 5.1D) that also demonstrated that neurons are the main source of NPY, since Tuj-1 immunoreactivity (neuronal marker) co-localized with NPY labeling (Figure 5.1D), whereas there were co-localization of NPY with specific markers for astrocytes or microglia (GFAP or CD11b labeling, respectively; data not shown).

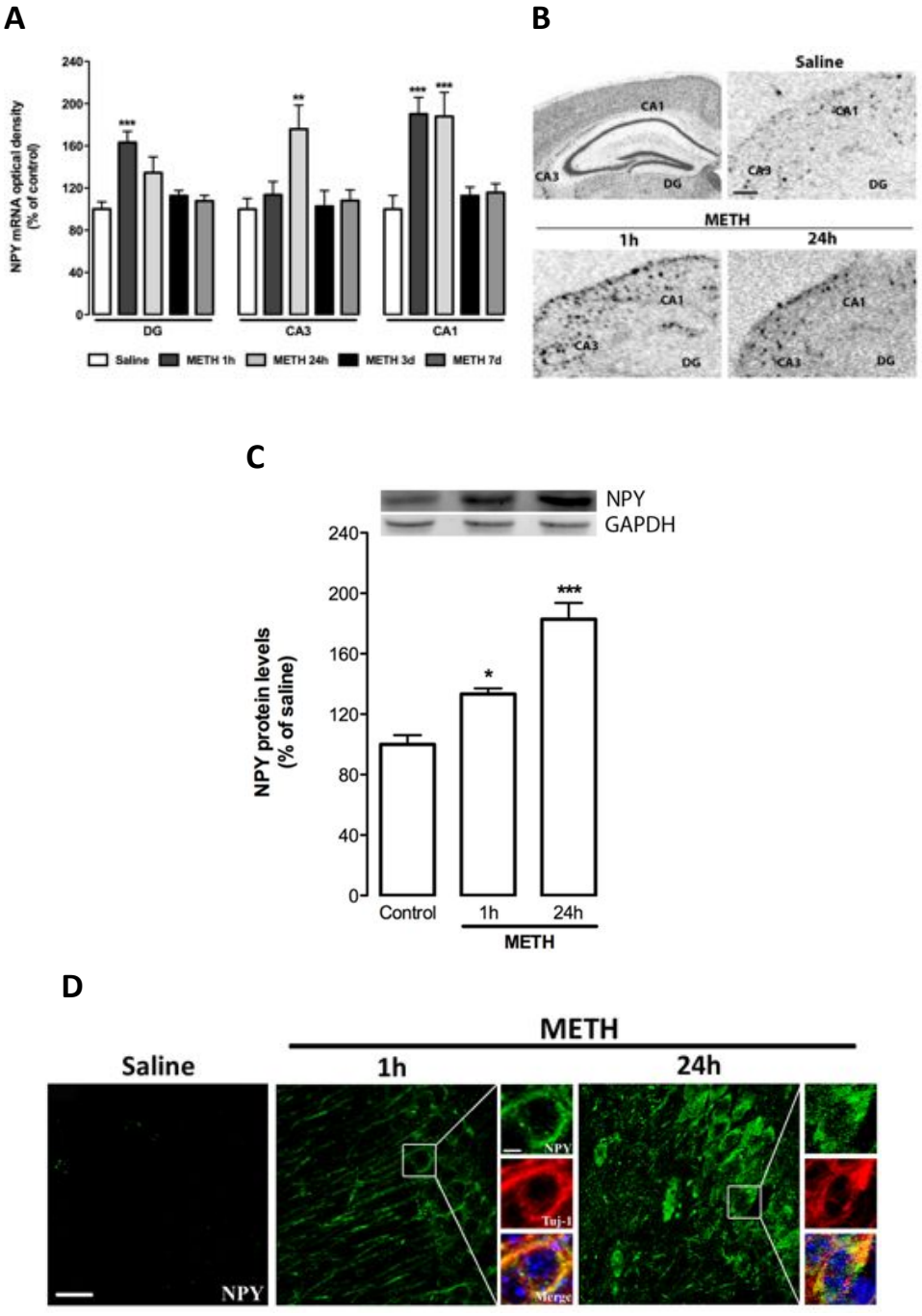
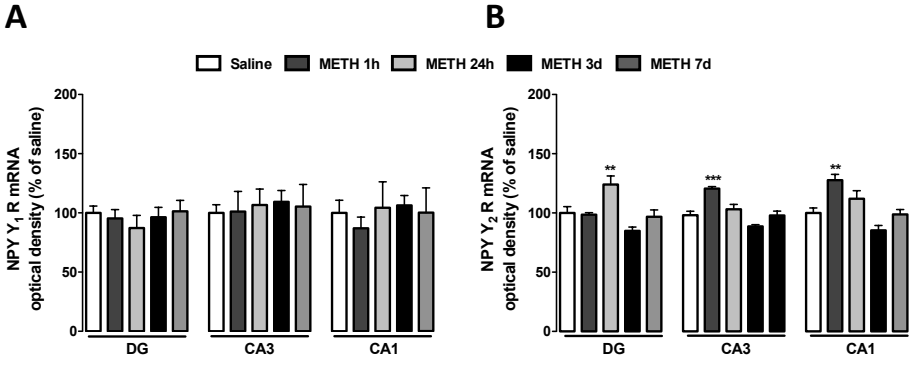


Figure 5.1. *METH increases NPY mRNA and protein levels in the mouse hippocampus. (A) Quantification of NPY mRNA levels in the hippocampal formation and (B) representative* in situ *hybridization autoradiograms. An image from hippocampus with Nissl staining (adapted from Sidman and collaborators, High Resolution Mouse Brain Atlas; www.hms.harvard.edu) is shown to facilitate the visualizing of hippocampal anatomy. The results are expressed as mean percentage of saline ± SEM (n = 6-10);* $^{**}p< 0.01$, $^{***}p< 0.001$, *significantly different from the*

respective saline sub-region. (C) METH also increased NPY protein levels at 1h and 24h post-administration. Above the bars, representative western blots for NPY (15 kDa) and GAPDH (34 kDa) are shown. Data are presented as mean percentage of saline ± SEM (n = 4-9); $^{*}p< 0.05$, $^{***}p< 0.001$, when compared to the saline. (D) Representative images of NPY immunoreactivity (green) in the CA1 pyramidal cell layer. Scale bars = 20 µm or 5 µm (detail images).

Since NPY in the hippocampus acts predominantly *via* Y_1, Y_2 and Y_5 receptors (see review Silva *et al*. 2005a; Xapelli *et al*. 2006), we also examined whether METH was able to induce changes in the receptor mRNA levels. We concluded that METH did not induce significant changes in Y_1 mRNA levels (Figure 5.2A). However, there was a significant increase of Y_2 mRNA levels in CA3 (120.6 ± 1.7% of control, n=6; $p < 0.0001$) and CA1 (127.6 ± 5.0% of control, n=7; $p < 0.0001$) pyramidal cells at 1h post-injection (Figure 5.2B), and in dentate granule cells after 24h (124.0 ± 7.3% of control, n=8; $p < 0.0001$; Figure 5.2B). Accordingly, we also observed an up-regulation of Y_2 receptor protein levels after 1h in both CA3 and CA1 sub-regions (Figure 5.2D). At 3d and 7d post-drug administration the values returned to basal levels. Moreover, we observed an increase of Y_5 receptor mRNA levels in all hippocampal sub-regions analyzed and at 1h after METH treatment as follows: DG, 159.8 ± 17.8% of control (n=7; $p < 0.0001$); CA3, 146.3 ± 16.7% of control (n=7; $p < 0.0001$); CA1, 158.7 ± 15.2% of control (n=6; $p = 0.059$) in CA1 (Figure 5.2C).



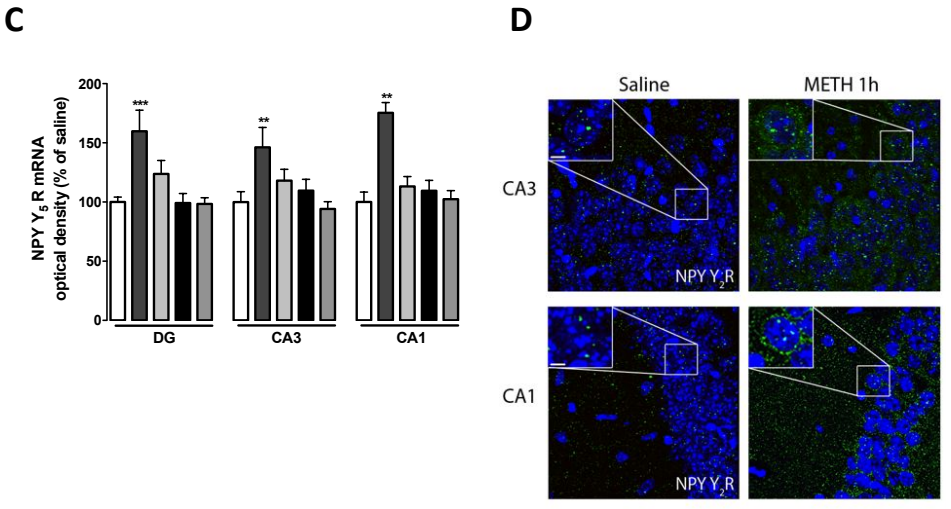


Figure 5.2. *Effect of METH on NPY receptor levels. (A) Y_1 receptor gene expression is unchanged while (B) Y_2 and (C) Y_5 receptor mRNA levels are increased in the mouse hippocampus by METH. The results are expressed as mean percentage of saline ± SEM (n = 6-12); $^{**}p< 0.01$, $^{***}p< 0.001$, significantly different from the respective saline sub-region. (D) Representative fluorescence images showing the increase of NPY Y_2 receptor protein levels (green; Hoechst 3342 - blue) in the CA3 and CA1 pyramidal cell layers at 1h post-METH administration. Scale bars = 20 µm or 5 µm (detail images).*

5.4.2. Changes in NPY receptor binding induced by METH

The previous findings led us to hypothesize that the activity of NPY receptors could be also affected by METH. In fact, we demonstrated that total [^{125}I]-PYY binding, which allows us to visualize the total NPY binding sites (Sovago *et al.*, 2001), was significantly increased in the CA1 sub-region at 1h following METH injection (1074.0 ± 82.5 pCi/mol, n=7; p = 0.043) when compared with saline (736.0 ± 52.1 pCi/mol, n=9; Figure 5.3A and B). However, no significant changes were detected in DG (p = 0.32) and CA3 subfields (p = 0.74; Figure 5.3A and B). No differences were detected in non-specific binding between saline and METH-treated mice (data not shown).

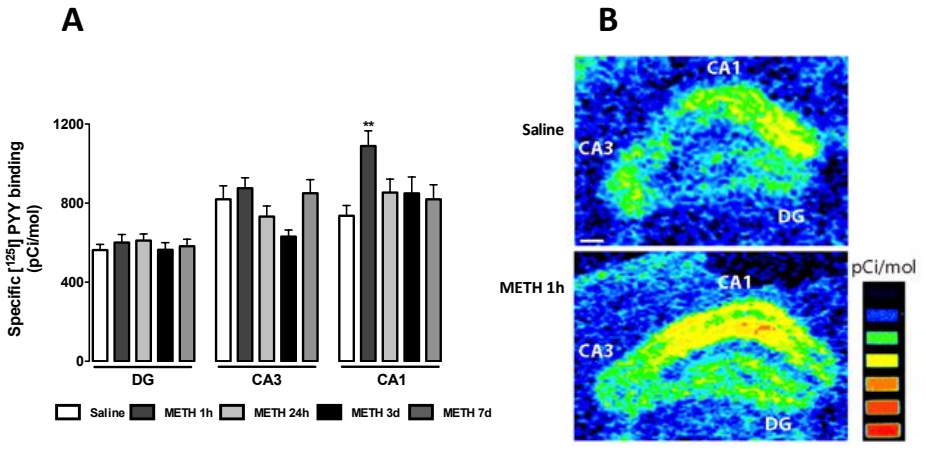


Figure 5.3. *Total NPY binding is increased in the CA1 sub-region at 1h following METH treatment. (A) Quantification of specific [^{125}I]PYY binding in the mouse hippocampal DG, CA3 and CA1 sub-regions. The results are expressed as mean pCi/mol ± SEM (n =7-13); $^{**}p< 0.01$, significantly different from the respective saline sub-region. (B) Representative pseudocolor autoradiograms of the hippocampal formation of saline and METH-treated mice showing a significant increase of [^{125}I]PYY binding in the CA1 sub-region. Scale bar = 500 µm.*

Then, we evaluated the effect of METH on the functional binding of Y_1, Y_2 and Y_5 receptors using [^{35}S]GTPγS binding assay. We concluded that METH administration induced a down-regulation of specific Y_1 receptor-stimulated binding in the hippocampal formation at 1h as follows: DG, -6.2 ± 4.3 nCi/g, n=7, p = 0.0374; CA3, -21.1 ± 8.3 nCi/g, n=8, p = 0.0139; CA1, -27.0 ± 10.7 nCi/g, n=8, p = 0.005; Figure 5.4A). On the other hand, METH induced a significant increase in Y_2 receptor binding to 119.9 ± 14.8 (n=10), 154.1 ± 9.3 (n=7) or 148.3 ± 11.0 (n=7) pCi/g (p < 0.0001) at 1h, 24h or 3d post-injection in CA3 sub-region, respectively, without significant changes at 7d (Fig. 5.4B). Furthermore, in CA1 sub-field, METH led to an up-regulation of Y_2 receptor at 24h (143.7 ± 12.5 pCi/g, n=7) that remains until 3d post-drug (143.5 ± 5.3 pCi/g, n=7; p < 0.0001; Figure 5.4B). In the figure 5.4D is possible to observe the Y_2 receptor functional binding at 24h post-METH and the blockade by the Y_2 antagonist, BIIE0246. Besides, no changes were detected regarding Y_5 receptor functional binding after injection (Figure 5.4C). Taken together, these results indicate that METH not only interferes with the expression of NPY receptors but also their

functionality. Interestingly, Y_1, Y_2 and Y_5 are also differentially affected by this drug of abuse.

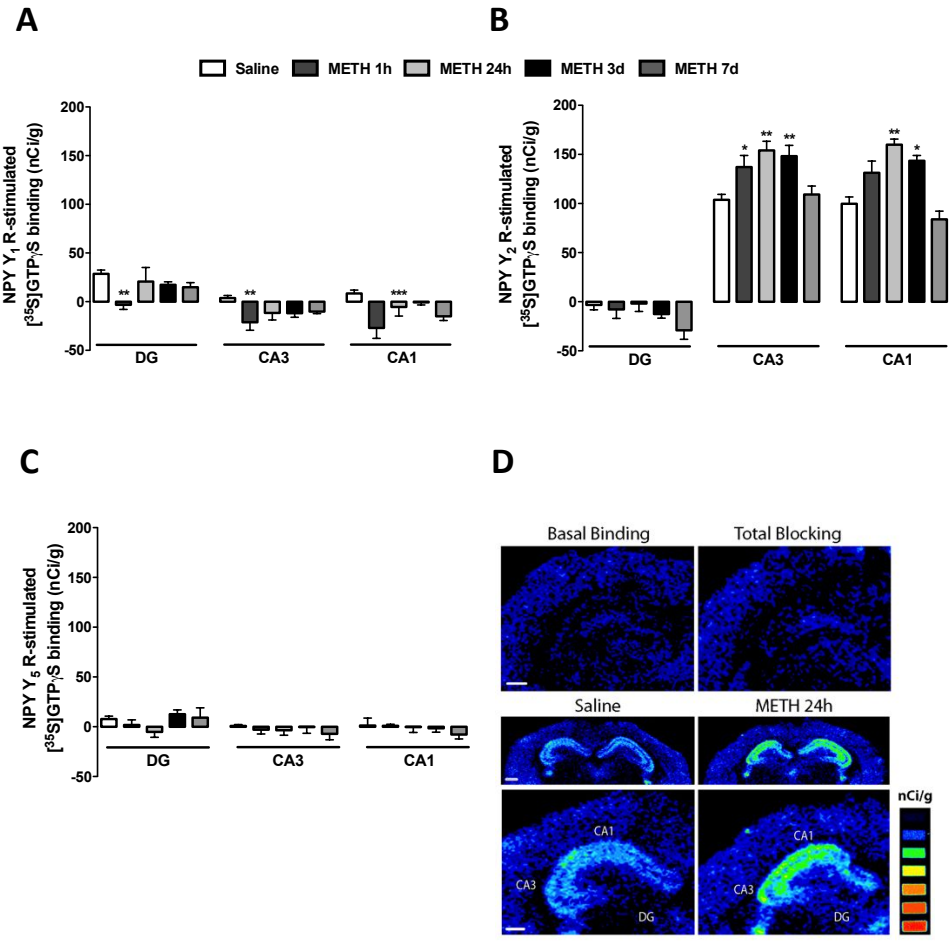


Figure 5.4. *Effect of METH on NPY receptor functionality. (A) Y_1 receptor-stimulated [^{35}S]GTPγS binding was decreased in the three hippocampal subfields analyzed at 1h post-METH. (B) Y_2 receptor specific functional binding was up-regulated in both CA3 and CA1 sub-regions, at 1h, 24h and 3d post-drug treatment. (C) No changes were observed in Y_5 receptor functional binding. The results are expressed as mean nCi/g ± SEM (n = 6-15); $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, significantly different from the respective saline sub-region. (D) Representative pseudocolor autoradiograms showing basal binding, total blocking, and bilateral increase of NPY Y_2 receptor-stimulated [^{35}S]GTPγS binding in the CA3 and CA1 sub-regions after 24h. Magnification of the left hippocampus autoradiograms in saline and METH-treated mice is also shown. Scale bar = 1 mm or 500 µm.*

5.4.3. Selective blockade of Y_2 receptors prevents memory impairment induced by METH

Since NPY plays an important role in several physiological functions including memory processing (Thorsell *et al.*, 2000; Karl *et al.*, 2008; Sorensen *et al.*, 2008a) and considering the changes of the hippocampal NPY system induced by METH, our next goal was to investigate if this drug of abuse led to memory deficits. For that, we examined spatial working memory performance using a Y-maze test and verified that METH-treated mice spent less time inside the novel arm (81.5±7.0, n=15, p = 0.0001) as compared to the saline-treated animals (119.6±4.3, n=14; Figure 5.5A). Taking in consideration that the integrity of hippocampus is not only essential to spatial working memory but also to recognition memory (Broadbent *et al.*, 2004), we also performed the novel object recognition test, and similarly to the previous results, the preference toward a novel object in the METH-treated mice decreased (44.2±3.7 recognition index, n=15; p = 0.0002) when compared to the saline group (66.3±2.1 recognition index, n=13; Figure 5.5B). Importantly, in Y-maze and novel object recognition test, the experimental groups showed no significant differences regarding the number of entries into the novel arm and the interaction with the objects, respectively, as well as no differences in the exploratory activity and motivation (data not shown). Also, mice never exhibited a stereotypic behavior during tests. Thus, we can conclude that acute METH treatment leads to memory deficits.

In order to disclose the possible correlation between NPY system changes, namely the NPY and Y_2 receptors up-regulation, and the memory deficits observed in the METH-treated mice, we evaluated if blockade of Y_2 receptors could prevent the recognition memory impairment. In fact, we verified that METH-induced memory impairment observed in both Y-maze and novel object recognition tests was completely prevented in the presence of the Y_2 receptor antagonist, BIIE0246 (113.7±8.6, n=10, p=0.008 and 63.3±4.2 recognition index, n=7, p = 0.0039; Figure 5.5A and B, respectively), suggesting that METH-induced memory deficits were mediated by the activation of Y_2 receptors. Importantly,

neither horizontal nor vertical exploratory activity was affected by combination of METH and BIIE0246 (data not shown), as previously demonstrated by Thorsell *et al.* (2002).

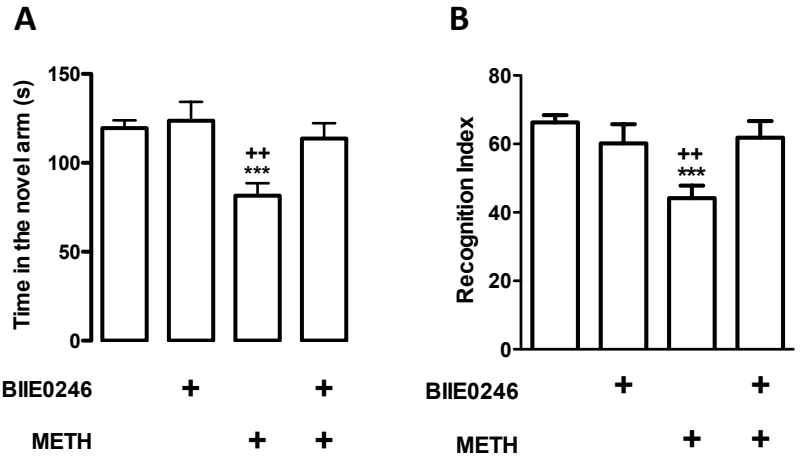


Figure 5.5. *METH impairs spatial and recognition memory in mice. (A) In the modified Y-maze test, mice treated with METH spent less time exploring the novel arm as compared to saline. The BIIE0246 pre-treatment completely prevented METH-induced spatial memory deficit. Data are presented as mean time spent in the novel arm ± SEM (n = 7-14) (B) The novel object recognition task shows impairment of recognition memory in METH-treated mice, and Y_2 receptor antagonist (BIIE0246) was able to block this effect. The results are expressed as mean recognition index ± SEM (n = 7-15).* $^{***}p< 0.001$*, when compared with saline;* $^{++}p< 0.01$*, significantly different from METH plus BIIE0246 treated mice.*

5.4.4. Modulation of AKT/mTOR signaling pathway by METH

Long-term synaptic plasticity and memory require protein synthesis, which is regulated by various signaling pathways (Kandel, 2001), including the AKT/mammalian target of rapamycin (mTOR) pathway (Hoeffer & Klann, 2010). Concerning the memory impairment induced by METH, we further investigated if this drug could also disrupt the AKT/mTOR pathway. We observed that METH significantly decreased the phosphorylation levels of AKT, mTOR and its p70S6k (Figure 5.6). Specifically, at 1h post-METH there was a decrease in AKT phosphorylation levels (76.6±3.2% of control, n=10; p = 0.0001; Figure 5.6A) that was confirmed by immunohistochemical analysis, showing that phospho-

AKT (p-AKT) immunolabelling reduction in the CA3 (Figure 5.6B), DG and CA1 (data not shown) sub-fields. Accordingly, we also observed a significant decrease of p-mTOR and p-p70S6k levels at 1h (57.2±6.1% of control, n=13; *p* = 0.0044; Fig. 5.6C) and 24h (73.3±4.1% of control, n=17; *p* = 0.0001; Fig. 5.6D), respectively, post-METH administration. Here, we also verified that Y_2 receptor blockade prevented the down-regulation of the phosphorylated levels of AKT (188.3±16.6% of control, n=5; *p* = 0.0020), mTOR (105.3±5.8, n=5; *p* = 0.0055) and p70S6k (92.7±3.7, n=7; *p* = 0.0047), as shown in figures 5.6A-D. Altogether, these findings show that METH-induced memory impairment was accompanied by a disruption of the AKT/mTOR signalling pathway, which was once again prevented by the Y_2 receptor blockade.

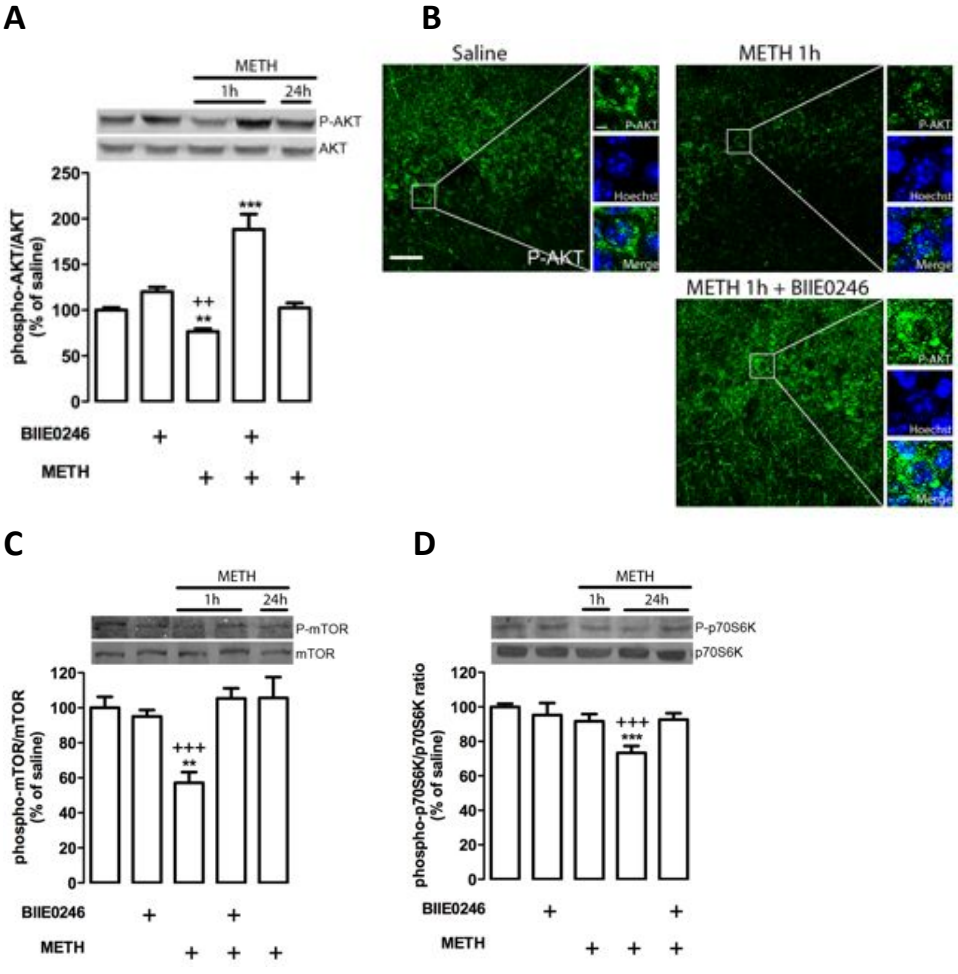


Figure 5.6. *METH modulates the AKT/mTOR pathway in the mouse hippocampus. (A,B) METH reduced the phosphorylation levels of AKT (60 kDa) or (C) mTOR (286 kDa) at 1h post-drug administration, and (D) p70S6K (70 kDa) phosphorylation levels at 24h post-METH. The Y_2 receptor antagonist (BIIE0246) was able to counteract this effect. (B) Representative images of phospho-AKT (green) immunoreactivity show a decrease in the CA3 pyramidal cell layer following 1h of METH injection, which was prevented by Y_2 receptor antagonist. Scale bars = 20 μm or 5 μm (detail images). Data are presented as mean percentage of saline ± SEM (n = 6-15); $^{**}p< 0.01$, $^{***}p<0.001$, when compared with saline; $^{++}p<0.001$, $^{+++}p<0.001$, significantly different from METH plus BIIE0246.*

5.5. Discussion

The present work reveals that hippocampal NPY system alterations are centrally involved in memory impairment induced by METH intoxication. Specifically, METH-induced memory deficit was abolished by Y_2 receptor blockade, and we further unraveled the involvement of the AKT/mTOR signaling pathway in these effects.

METH is an illicit psychostimulant drug that affects several brain regions leading to neurological changes that include damage to monoaminergic neurons, grey matter loss, white matter hypertrophy and excitotoxicity (reviewed by Cadet & Krasnova, 2009). Recently, we verified that an acute METH treatment leads to astrogliosis, microglia activation and tumor necrosis factor-alpha (TNF-α) production, accompanied by neuronal dysfunction, namely disturbance in cytoskeletal, synaptic and axonal protein levels, but without evidence of hippocampal cell death (Gonçalves *et al.*, 2010). Furthermore, the same METH regimen increases the BBB permeability in the hippocampus, without any effect in the striatum and frontal cortex (Martins *et al.*, 2011), which led us to conclude that the hippocampus is particularly susceptible to METH. Concerning the impact of METH on the NPYergic system, Thiriet *et al.* (2005) showed that METH (4 x 10 mg/kg each 2h) increases striatal NPY mRNA levels after 3 and 7 days. Moreover, the same study reported that METH induced a down-regulation of NPY Y_1 receptor mRNA and a contrasting up-

regulation of Y_2 receptor mRNA (Thiriet *et al.*, 2005). In fact, neuroplastic changes in the NPY system are prominent in several brain pathologies, such as epilepsy, Parkinson's disease, brain ischemia or drug abuse (Goodman & Sloviter, 1993; Cannizzarro *et al.*, 2003; Duszczyk *et al.*, 2009; Olling *et al.*, 2009).

Here, we verified that expression levels of NPY and its receptors, as well as receptor function, were differentially altered by METH depending on hippocampal sub-region and time-point analyzed. Accordingly, other studies have demonstrated that distinct insults differentially modulate the NPYergic system. Olling *et al.* (2009) described that ethanol-treated rats (5 x 20% ethanol daily, during 4 days) only changed the NPY Y_2 receptor mRNA levels in DG sub-region. In contrast, repeated electroconvulsive seizures lead to an up-regulation of NPY Y_1, Y_2 and Y_5 receptor mRNA levels in DG sub-field together with a decrease in Y_2 receptor mRNA levels in CA3 cell layer of mice hippocampus (Christensen *et al.*, 2006). NPY receptor localization in the hippocampal sub-regions may be one possible explanation for these differences. In fact, the Y_1 receptor is highly expressed in DG, Y_2 receptor shows a strong expression in CA3 and CA1 pyramidal cell layers and Y_5 receptor is mainly expressed in DG and CA1 sub-fields (Naveilhan *et al.*, 1998). Several authors have suggested that NPYergic system changes can be an adaptive mechanism for counteracting degeneration and/or cell death (reviewed by (Vezzani & Sperk, 2004); Xapelli *et al.*, 2006), but NPY signaling is not only involved in neuroprotection. Indeed, there are studies showing that NPY receptors regulate voluntary ethanol consumption and its toxic effects (Thiele *et al.*, 2002; Rimondini *et al.*, 2005; Schroeder *et al.*, 2003). Moreover, the activation of Y_1 receptors can have proconvulsive effects (Olesen *et al.*, 2012), while Y_2 receptors revealed an important role in the generation of anxiety- and stress-related behaviors in mice (Tschenett *et al.*, 2003).

Multiple strands of evidence indicate an important role for the hippocampus in the formation and retrieval of episodic and contextual memories in humans and animals (Wiltgen *et al.*, 2010; Vann & Albasser, 2011),

and it is well established that hippocampal dysfunction produces pronounced amnesia for newly acquired information (reviewed by (Frankland & Bontempi, 2005). Our group previously described that acute subcutaneous injection of 30 mg/kg METH impairs rat spatial working memory, one of the hallmarks of hippocampal integrity (Simões *et al.*, 2007). Besides, it has been demonstrated that extended access to METH produces deficits in both spatial and recognition memory in rodents (Belcher *et al.*, 2008; Lee *et al.*, 2011; O'Dell *et al.*, 2011; Reichel *et al.*, 2011) that are comparable to those registered in human METH abusers (Scott *et al.*, 2007). It is known that the storage and update of memory require hippocampal plasticity mechanisms, such as LTP (see review Bruel-Jungerman *et al.*, 2007; Neves *et al.*, 2008). In fact, it was shown recently that rats (Hori *et al.*, 2010) and mice (Swant *et al.*, 2010) chronically exposed to METH exhibited a decrease of membrane potential and in the LTP magnitude in the CA1 pyramidal cell layer. So, it is plausible to hypothesize that defective hippocampal synaptic plasticity could trigger METH-induced cognitive performance deficits. Moreover, it is known that METH consumption interferes with several neurotransmitters, including glutamate (reviewed by Krasnova & Cadet, 2009; Silva *et al.*, 2010) that plays a pivotal role in memory function, such as induction of LTP (reviewed by Bliss & Collingridge, 1993). In fact, METH-injected rats (7.5 mg/kg every 2h over a period of 6h; i.p.) exhibited augmented hippocampal extracellular glutamate levels (Raudensky & Yamamoto, 2007), and we previously demonstrated that an acute METH treatment (30 mg/kg, s.c.) leads to up-regulation of NMDA and AMPA receptors in the rat hippocampus (Simões *et al.*, 2007). Here, we describe that METH increases the levels of hippocampal NPY and Y_2 receptors, as well as Y_2-mediated functional activity. Moreover, it was previously shown that activation of presynaptic Y_2 receptors mediates NPY-induced inhibition of hippocampal glutamate release (Silva *et al.*, 2001), which can explain LTP compromising at CA1-subicular cell synapses (Sørensen *et al.*, 2008a). Thus, we hypothesized that the changes verified here in the hippocampal NPY system could underlie METH-induced mnemonic deficits. The contribution of NPY and its receptors to cognitive behaviors has

not been much explored. Nevertheless, Thorsell *et al.* (2000) demonstrated that transgenic rats overexpressing NPY in hippocampus show spatial memory impairment. Supporting this evidence, it was shown that vector-mediated hippocampal NPY overexpression in rats impaired LTP in the CA1 subfield and, consequently, the animals exhibited a delay in hippocampal-dependent spatial discrimination learning (Sørensen *et al.*, 2008b). Interestingly, in these two transgenic models the overexpression of NPY was accompanied by decreased NPY Y_1 receptor binding, without alterations of NPY Y_2 receptor activity (Thorsell *et al.*, 2000; Sørensen *et al.*, 2008b). Moreover, we observed an early up-regulation in both mRNA and protein levels of Y_2 receptors, as well as in their activity that remained altered until 3d post-METH injection, which was significant in the CA1 sub-region that has a prominent role in hippocampal plasticity. Thus, our data suggest that NPY and Y_2 receptor changes are involved in memory deficits induced by METH, since we further demonstrated that the blockade of Y_2 receptors completely prevented METH-induced memory impairment.

Synaptic plasticity, and consequently memory formation, requires new protein synthesis (Kandel, 2001; Bruel-Jungerman *et al.*, 2007; Costa-Mattioli *et al.*, 2009). Furthermore, protein synthesis underlying the formation of long-lasting memories is highly regulated at the translation level, where the AKT/mTOR pathway regulates the translation rate and the integration of information from diverse synaptic inputs (reviewed by Hoeffer & Klann, 2010). Indeed, AKT/mTOR signaling cascade was identified as being crucial to the induction of protein synthesis-dependent synaptic plasticity required for the hippocampus-dependent learning and memory processes (Cammalleri *et al.*, 2003; Opazo *et al.*, 2003). Hence, to unravel the signaling pathways mediating the anti-mnemonic effects of METH, we explored the potential involvement of the AKT/mTOR pathway. We found that an acute dose of METH disrupted the activation of AKT/mTOR in the hippocampus, which underlies long-term memory impairment, through Y_2 receptor activation. Moreover, we have previously shown that NPY *via* Y_2 receptor activation inhibits Ca^{2+} influx (Silva *et*

al., 2003a), which, in turn, can restrain AKT/mTOR cascade compromising new protein synthesis and, consequently, inhibiting LTP (Opazo *et al.*, 2003b). Indeed, the impairment of protein synthesis machinery and LTP results in memory deficits. Thus, it is reasonable to postulate that our observations could be explained by Ca^{2+} depression mediated by activation of the hippocampal NPY system. Importantly, we have considered the use of knockout (KO) mice to complement this study, but we identified several problems both in Y_2 (Zambello *et al.*, 2011) and AKT (Chen *et al.*, 2001; Woulfe *et al.*, 2004; Easton *et al.*, 2005) KO mice that led us to exclude its use for this specific purpose. Thus, we decided to use a pharmacology approach by applying the Y_2 receptor antagonist, BIIE0246 (Dumont *et al.*, 2000).

In summary, the present study provides a new plausible explanation for the memory deficits induced by METH intoxication. Our work supports the potential importance of the hippocampal NPY system, and the functional relationship between Y_2 receptors and AKT/mTOR pathway activation in memory impairment induced by METH. Thus, we may suggest that targeting this pathway or the modulation of Y_2 receptor activity could provide interesting therapeutic approaches.

Chapter 6

Protective role of neuropeptide Y Y_2 receptors in cell death and microglial response following methamphetamine injury

6.1. Abstract

It has been reported that hippocampus is very susceptible to methamphetamine (METH) and that neuropeptide Y (NPY) is an important neuroprotective agent against hippocampal excitotoxicity. However, there is very little information regarding the role of NPYergic system in this brain region under conditions of METH toxicity. To clarify this issue, we investigated the role of NPY and its receptors against METH-induced neuronal cell death in hippocampal organotypic slice cultures. Our data show that NPY (1 μM) is neuroprotective in DG, CA3 and CA1 sub-regions *via* Y_2 receptors. Moreover, the selective activation of Y_1 receptors (1 μM [Leu^{31},Pro^{34}]NPY) partially prevented the toxicity induced by METH in DG and CA3 sub-fields, but completely blocked its toxicity in the CA1 pyramidal cell layer. Regarding Y_2 receptors, its activation (300 nM NPY13-36) completely prevented the METH toxicity in all sub-regions analyzed, which involved changes in pro- and anti-apoptotic protein levels, Bcl-2 and Bax, respectively. Besides neuronal cell death, we also showed that METH triggers microglial reponse in mice hippocampus, which was attenuated by Y_2 receptor activation. To better clarify the effect of this drug and the NPY system on microglial cells, we further used the N9 microglial cell line. We found that both NPY and the Y_2 receptor agonist were able to protect microglia against METH-induced cell death. Overall, our data demonstrate that METH is toxic to both neurons and microglial cells, and that NPY, mainly *via* Y_2 receptors, has an important protective role against METH-induced cell death and microgliosis.

6.2. Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide abundant in the mammalian central nervous system (CNS) (Silva *et al.*, 2005a; Xapelli *et al.*, 2006), being an important neuromodulator in the hippocampus, where exert its biological functions *via* Y_1, Y_2 and Y_5 receptor subtypes (Gehlert, 2004). Within the hippocampus, it has been described that NPY receptors are differently distributed. In fact, the Y_1 and Y_5 sub-types are highly expressed in the DG sub-region, especially in dendrites processes of granular cells and interneurons (Caberlotto *et al.*, 1997; Nichol *et al.*, 1999; Grove *et al.*, 2000; Sperk *et al.*, 2007). Also, Y_1 receptors are mostly present in glutamate-positive cells, whereas the Y_5 receptor immunoreactivity appears in gamma-aminobutyric acid (GABA)-positive cells (Grove *et al.*, 2000; Sperk *et al.*, 2007). On the other hand, the Y_2 receptors are more present in the CA3 and CA1 sub-fields being mainly expressed by GABA- and glutamate decarboxylase 67 (GAD67)-positive pyramidal neurons (Caberlotto *et al.*, 1998; Sperk *et al.*, 2007). Regarding the presence of NPY receptors in hippocampal glial cells, only one study has explored this issue by demonstrating that Y_1 receptors co-localize with glial fibrillary acidic protein (GFAP)-positive hippocampal astrocytes (St-Pierre *et al.*, 2000). NPY is involved in many biological events like appetite and circadian rhythms regulation (Berglund *et al.*, 2003), cognitive processing (Karl *et al.*, 2008; Sorensen *et al.*, 2008a) and seizure activity modulation (Silva *et al.*, 2005a; Woldbye *et al.*, 2005; Christensen *et al.*, 2006). Moreover, the NPYergic system can be altered in several neuropathologies, such as epilepsy, Parkinson´s disease and ischemia (Cannizzaro *et al.*, 2003; Silva *et al.*, 2005a; Christensen *et al.*, 2006; Duszczyk *et al.*, 2009). Indeed, we and others reported that NPY has a crucial neuroprotective role against hippocampal excitotoxicity (Silva *et al.*, 2003b; Xapelli *et al.*, 2007, 2008; Álvaro *et al.*, 2009; Woldbye *et al.*, 2010; Gotzsche *et al.*, 2011; Olesen *et al.*, 2012). Specifically, *in vitro* studies showed that both Y_1 and Y_2 receptors activation were able to rescue post-kainate (KA) insult injured hippocampal neurons (Silva *et al.*, 2003b; Xapelli *et*

al., 2007). In animal models of epilepsy it was described that hippocampal Y_1 receptor overexpression aggravates seizures, while Y_2 receptor overexpression suppresses it (Woldbye *et al.*, 2010; Olesen *et al.*, 2012). Despite the well known beneficial role played by the NPY system under several pathological conditions, little information is available regarding its role under conditions of brain toxicity induced by drugs of abuse (Goodman & Sloviter, 1993; Thiele *et al.*, 2004; Thiriet *et al.*, 2005; Horner *et al.*, 2006; Olling *et al.*, 2007).

Methamphetamine (METH) is an addictive psychostimulant drug that causes irreversible brain damages leading to neurological and psychiatric anomalies (Thompson *et al.*, 2004; Nakama *et al.*, 2008; Kish *et al.*, 2009; Kitamura *et al.*, 2010). METH-induced neurotoxicity involves several cellular events like dopamine oxidation (Hadlock *et al.*, 2009; Kita *et al.*, 2009), oxidative stress (Kita *et al.*, 2009; Yamamoto *et al.*, 2010) and release of high levels of glutamate resulting in excitotoxicity (Smith *et al.*, 2010). In addition, it was demonstrated that multiple high doses of METH increases the levels preproNPY mRNA in rat striatal neurons (Horner *et al.*, 2006). Thiriet and collaborators (2005) also verified that METH repeated treatment induces up-regulation of striatal NPY mRNA, and down-regulation or biphasic changes in Y_1 or Y_2 receptor expression, respectively. Moreover, this work showed that NPY *via* Y_1 and Y_2 receptor activation decreases METH-induced striatal neurotoxicity (Thiriet *et al.*, 2005). Recently, our group reported that METH intoxication induces hippocampal neuroinflammation, neuronal dysfunction and blood-brain barrier (BBB) disruption, without causing cell death (Gonçalves *et al.*, 2008, 2010; Martins *et al.*, 2011). Also, we verified that METH negatively affects DG cell viability and neurogenesis, and activation of Y_1 and Y_2 receptors was able to prevent METH-induced dentate gyrus (DG) cell death (Baptista *et al.*, 2012).

Several studies have revealed that hippocampus is particularly susceptible to METH, but the information about the role of NPY under METH-induced toxicity is scarce. So, with the presence study we aimed to evaluate the

possible protective role of NPY and its receptors against METH-induced hippocampal cell death and microgliosis.

6.3. Materials and Methods

6.3.1. Mouse hippocampal organotypic slice cultures

Hippocampal slice cultures were prepared from 6-8 day-old wild type C57BL/6 mice according to the interface culture method (Stoppini *et al.*, 1991) and as previously described by us (Bernardino *et al.*, 2005b; Xapelli *et al.*, 2007, 2008). Specifically, mice were sacrificed, brains were removed rapidly under sterile conditions, and both hippocampi were isolated and cut in transverse slices with 350-µm-thick using a McIlwain tissue chopper. The slices were placed in ice-cold Gey's balanced salt solution (GBSS; Sigma-Aldrich St. Louis, MO, USA) with 25 mM D-glucose (Merck, Darmstadt, Germany) and 0.01% Penicillin-Streptomycin (Invitrogen, Paisley, UK), and the excess of tissue was removed. Afterwards, individual slices were placed on porous (0.4 µm) insert membranes (Millipore, Madrid, Spain) with a total of six slices in each membrane, and transferred to six-well culture trays (Orange Scientific, Braine-l'Alleud, Belgium). Each well contained 1 ml culture medium composed of 50% Opti-MEM, 25% heat-inactivated horse serum, 25% Hank's balanced salt solution (HBSS) and 0.01% Penicillin-Streptomycin (all from Invitrogen), supplemented with D-glucose to a final concentration of 25 mM (Merck). Cultures were maintained in an incubator with 5% CO_2 and 95% atmospheric air at 37°C with medium change twice each week for the following two weeks. Before starting the experiments, culture medium was replaced by serum-free Neurobasal medium (Invitrogen), supplemented with 1 mM L-glutamine (Sigma-Aldrich), 2% B-27 supplement and 0.01% Penicillin-Streptomycin (Invitrogen).

Experimental procedures were performed according to the guidelines of the European Community Council Directives (86/609/EEC) and the

Portuguese law for the care and use of experimental animals (DL 129/92). The present study was approved by Foundation for Science and Technology (PTDC/SAU-FCF/67053/2006). All efforts were made to minimize animal suffering and to reduce the number of animals.

6.3.2. Assessment of neuronal cell death

Neuronal cell death was assessed by monitoring the propidium iodide uptake (PI; 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide; Sigma-Aldrich) as described previously by us (Silva *et al.*, 2003b; Bernardino *et al.*, 2005b; Xapelli *et al.*, 2007, 2008). Three hours before starting experiments, 2 μM PI was added to the medium for determination of basal cellular uptake, and this concentration was also used in all subsequent medium changes. Cellular PI uptake was recorded by fluorescence microscopy (Axioskop 2 Plus, Carl Zeiss, Göttingen, Germany) using a standard rhodamine filter and a digital camera (AxioCam HRc, Carl Zeiss). Digital fluorescent micrographs were taken before drug exposure (basal PI uptake), and at fixed time-points after drug exposure. Then, PI uptake was measured by delineating the dentate gyrus (DG), *cornus ammonis* 3 (CA3) and 1 (CA1) hippocampal sub-regions using NIH ImageJ 1.44 analysis software. The effect of treatments was assessed by subtracting the basal PI uptake value (day 0, non-treated cultures) from the corresponding value after treatment (day 1 or day 2).

6.3.3. Experimental treatments in hippocampal organotypic slice cultures

Hippocampal slice cultures were incubated with different concentrations of METH [(+)-Methamphetamine hydrochloride; Sigma-Aldrich] (0.1, 0.2, 0.5, 1 and 10 mM) for 24h (Figure 6.1). Importantly, the concentration range of METH used in the present study was selected in accordance with prior *in vitro* studies (Aizenman *et al.*, 2010; Nopparat *et al.*, 2010; Nakagawa *et al.*, 2011). To test the role of NPY or NPY ligands, slice cultures were pre-incubated with 1 μM NPY

or with the selective Y_1 or Y_2 receptor agonists, 1 μM [Leu^{31},Pro^{34}]NPY or 300 nM NPY(13-36), respectively (Silva *et al.*, 2003b), during 24h (all from Bachem AG, Bubendorf, Switzerland). Then, cultures were exposed for more 24h with the respective ligands plus 500 μM METH (Figure 6.1). The same experimental protocol was used to clarify the selective effect of the NPY receptor agonists by using 1 μM BIBP3226 or 1 μM BIIE0246 (Tocris Bioscience, Bristol, UK), Y_1 or Y_2 receptor antagonist, respectively (Silva *et al.*, 2003b).

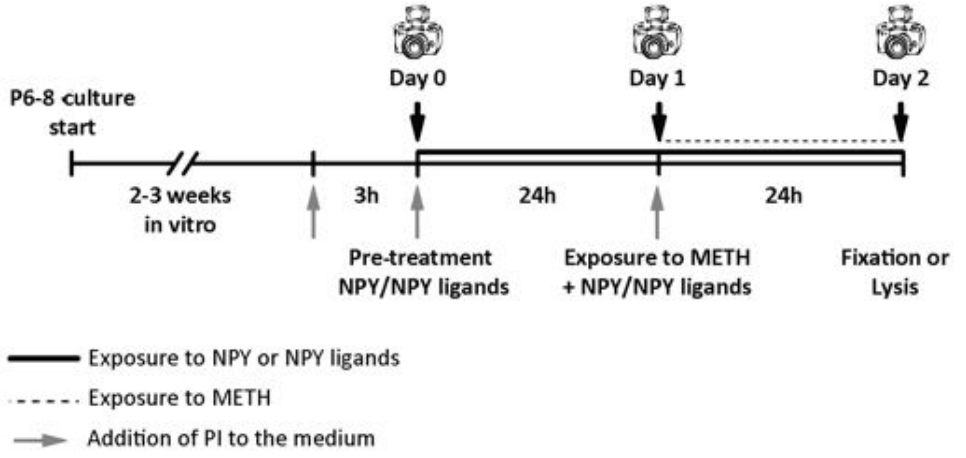


Figure 6.1. *Schematic representation of the experimental protocol to investigate the potential protective effect of NPY or its receptors against METH-induced cell death in mouse hippocampal organotypic slice cultures. For details, see text.*

6.3.4. RT-PCR analysis

The mRNA was isolated using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cultured slices without treatments were first lysed in a highly denaturing buffer containing guanidine thiocyanate, which ensured the inactivation of RNases. Then, the samples were applied to spin columns, where total RNA bound to the membrane, and the exclusion of contaminants and small size RNA allowed the purification of a high quality mRNA-enriched solution. Before the samples were quantified by measuring the ratio of OD at 260 and 280 nm using RNA/DNA calculator GeneQuant II (Pharmacia Biotech Amersham Biosciences AB, Uppsala, Sweden),

RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water (Fluka, Sigma-Aldrich). Total mRNA (2 μg) was transcribed using Oligo dT(15) primers (Bioron, Ludwigshafen, Germany), and PCR reaction was performed in a total volume of 50 μl containing 5 μl cDNA, 1 μl dNTP mix, 5 μl 10x complete buffer for Taq DNA polymerase, 0.25 μl Taq DNA polymerase (all from Bioron), 0.2 μl of both forward and reverse primers, and a variable volume of water. The primer sequences used were described in Table 6.1 (all presented as 5'-3': MWGBiotech AG, Ebersberg, Germany). The mRNA levels of β-actin were used as a loading control, and negative controls were performed to ensure that PCR products result from RNA transcription (data not shown). PCR products were separated by gel electrophoresis (1.5% agarose) and stained with ethidium bromide and densitometric analysis was performed on Universal Hood II (Bio-Rad Laboratories, Milan, Italy).

Table 6.1. *List of primers used in RT-PCR*

	Primers	
	Forward	**Reverse**
NPY	AGAGATCCAGCCCTGAGACA	AACGACAACAAGGGAAATGG
Y_1	AAATGTGTCACTTGCGGCGTTC	AGTGTTGATTCGCTTGGTCTCACTG
Y_2	GTAAGCCAAGTGAGAGTGA	CCATTTTCCACTCTCCCATCA
β-actin	GACTACCTCATGAAGATCCT	ATCTTGATCATGGTGCTG

6.3.5. Western blot analysis

Hippocampal organotypic slices were homogenized in ice-cold lysis buffer [150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 10% Triton X-100, 10% DOC, 10% sodium dodecyl sulphate (SDS), 100 mM dithiothreitol (DTT), pH 7.5; all from Sigma-Aldrich] containing Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, West Sussex, UK), and centrifuged at 14500 rpm for 20 min. Supernatants were stored at -80°C until further use. Protein concentration was determined using the BCA protein assay and equal amounts (50 μg per lane) were loaded and separated on SDS-polyacrylamide gel. Afterward, proteins

were transferred to polyvinylidene difluouride membranes (PVDF; Immobilon Transfer Membrane, Millipore, Bedford, MA). Membranes were blocked and incubated overnight (ON) at 4°C with the primary antibody mouse anti-Bcl2 (1:200; ref. sc-16321) or rabbit anti-Bax (1:100; ref. sc-6236; both from St. Cruz Biotechnology, Inc, Heidelberg, Germany). After rinses, membranes were incubated with the anti-mouse (1:10000; #NIF1316) or anti-rabbit (1:20000; #NIF1317) HRP-conjugated secondary antibodies (both from Amersham Bioscience, GE Healthcare Life Science, Barcelona, Spain) for 1h at room temperature (RT). The signal was obtained using the enhanced chemifluorescence substrate (ECL; Amersham Bioscience) and visualized in a Thyphoon FLA 9000 (GE Healthcare BioSciences AB, Uppsala, Sweden). Immunoblots were reprobed with mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000; ab9484, Abcam, Cambridge, UK) to ensure equal sample loading, and densitometric analyses were performed using the ImageQuant software.

6.3.6. Immunohistochemistry

Hippocampal slice cultures were fixed with ice-cold 4% paraformaldehyde (PFA; Merck) in 0.01 M phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4; all from Merck) at RT for 30 min, followed by cryopreservation in 30% sucrose and stored at 4ºC until further use. The slices were rinsed in 0.01 M PBS, and then blocked with 3% fetal bovine serum (FBS; Gibco) plus 2.5% Trinton X-100 (Sigma-Aldrich) in 0.01 M PBS for 1h 30min at RT, and incubated with rat anti-mouse CD11b antibody (1:500; ref. MCA71, AbD Serotec, Oxford, UK) at 4ºC during 24h. Afterwards, sections were incubated with goat anti-rat Alexa Fluor 488 antibody (1:200; A11006, Invitrogen) for 1h 30 min at RT, washed, and incubated with Hoechst 33342 (5 μg/ml; ref. 14533, Sigma-Aldrich) for 5 min at RT and, finally, mounted in Dako fluorescence medium (Dako North America, Carpinteria, USA). Z-stacks images were acquired using a LSM 710 Meta Confocal microscope (Carl Zeiss, Oberkochen, Germany). The quantification of fluorescence intensities of CD11b

antibody labeling was performed using the NIH ImageJ 1.44 analysis software. For that, a region was drawn around each cell to be measured, as well as other regions in an area without fluorescent objects to be used for background subtraction. To determine the corrected total cell fluorescence, we used the following formula: correct total cell fluorescence of each cell = integrated intensity - (area for the selected cell x mean background), as previous described (Gavet & Pines, 2010a, 2010b; Potapova *et al.*, 2011).

6.3.7. Microglial cell line culture

N9 murine microglial cells (kindly provided by Professor Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy) were cultured in T-25-cm^2 culture flasks in RPMI 1640 medium without glucose (Gibco, Paisley, UK) and supplemented with 30 mM glucose (Sigma-Aldrich), 23.8 mM $NaHCO_3$ (Sigma-Aldrich), 5% FBS (Gibco), 100 U/ml penicillin and 100 µ/ml streptomycin (Gibco). Cells were kept at 37°C in a 95% atmospheric air and 5% CO2 humidified atmosphere.

6.3.8. TUNEL assay

Microglial cells were plated in 24-well trays at a density of $4x10^4$ cells/ml and treatments were performed as shown in Figure 6.2. Specifically, cells were pre-exposed to 1 µM NPY or to the selective Y_1 or Y_2 receptor agonists, 1 µM [Leu^{31},Pro^{34}]NPY or 300 nM NPY(13-36), respectively, during 24h (all from Bachem AG) followed by more 24h in the presence of 1 mM METH (Coelho-Santos *et al*., 2012). As previously described to organotypic hippocampal slice cultures, the same experimental protocol was used to clarify the selective effect of the NPY receptor agonists by using 1 µM BIBP3226 or 1 µM BIIE0246 (Tocris Bioscience), Y_1 or Y_2 receptor antagonist, respectively. After treatments, whole population of cells were fixed with 4% PFA and centrifuged at 10000 rpm for 5 min (Cellspin I, Tharmac GmbH, Waldsolms, Germany) to adhere to Superfrost® microscope slides (Thermo Scientific, Braunschweig, Germany). Cell death was assessed by terminal deoxynucleotidyl transferase dUTP nick-

end labeling (TUNEL) according to the manufacturer's instructions. Microglial cells were rinsed with 0.01 M PBS, permeabilized in 0.25% Triton X-100 solution for 30 min at RT, and incubated with terminal deoxynucleotidyl transferase buffer (provided in TUNEL kit) during 1h at 37ºC in a humidified chamber. Afterwards, cells were rinsed in TB buffer (300 mM NaCl and 300 mM sodium citrate), washed in PBS, incubated with Fluorescein Avidin D (1:100; ref. A-2001, Vector Laboratory, Peterborough, UK) followed by Hoechst 33342 staining (Sigma-Aldrich). Finally, slides were mounted in Dako fluorescence medium (Dako North America, Carpinteria, USA) and images were recorded using a camera Leica DMIRE2 incorporate on Leica fluorescence microscope Leica CTRMIC (Leica, Germany).

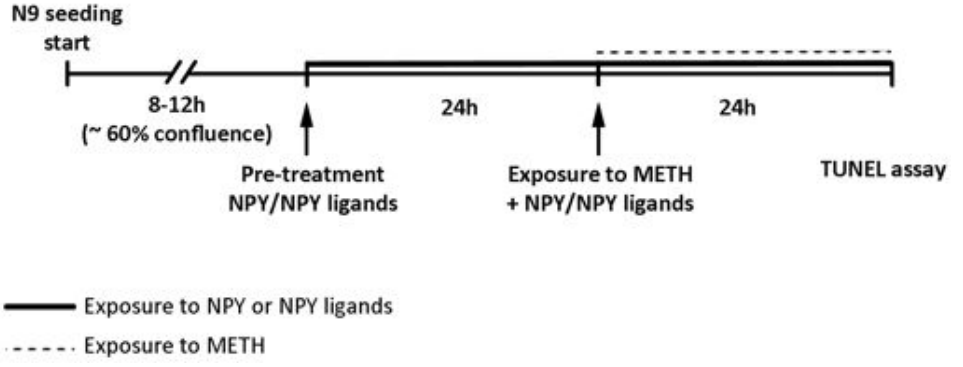


Figure 6.2. *Schematic representation of the experimental protocol to investigate the potential protective effect of NPY or its receptors against METH-induced cell death in N9 microglial cell line cultures. For details, see text.*

6.3.9. Statistical analysis

Data are expressed as mean values ± SEM. Statistics were performed using one-way ANOVA followed by Bonferroni's *post hoc* test or a Mann-Whitney test, as indicated in figure legend. Data were considered to be statistically different at $P < 0.05$. To evaluate the toxic effect of METH on hippocampal slice cultures, all the experimental conditions were compared with the respective control (drug-free condition), and the maximal PI uptake in each sub-region was set to 100%, and the EC_{50} value was calculated based on these curves. When

evaluating the effect of NPY or NPY ligands on METH-induced toxicity, the PI uptake values induced by 0.5 mM METH was set to 100%. Moreover, the independent samples (*n*) were defined to be the number of independent hippocampal slices obtained from at least 3 independent cultures. For TUNEL quantification, six independent microscopy fields were acquired per coverslip with 20x objective, and TUNEL-positive cells were counted and expressed as percentage of total cells stained with Hoechst 33342 per each field (*n* = microscopy field).

6.4. Results

6.4.1. METH-induced hippocampal neuronal cell death

Recently, we reported that a single high dose of METH triggers a neuroinflammatory response (Gonçalves *et al.*, 2008, 2010) and neuronal dysfunction in the hippocampus (Goncalves *et al.*, 2010). Moreover, the same protocol of METH administration induces an increase in BBB permeability only in the hippocampus (Martins *et al.*, 2011). Since our previous observations suggest that hippocampus is highly susceptible to METH, we further aimed to evaluate the toxic profile of this drug on hippocampal formation. For that, hippocampal organotypic slice cultures were either left untreated (control) or treated with increasing concentrations of METH, and we verified that 0.1 mM METH was not toxic (Figure 6.3), whereas 0.2 mM METH induced cell death in both DG and CA1 sub-regions (49.1±4.4%, $P = 0.001$, $n = 22$; and 48.7±6.4% of PI, $P = 0.0008$, $n = 22$, respectively; Figure 6.3). In fact, increasing concentrations of METH corresponded to increasing PI uptake in both DG and CA1 as follows: 0.5 mM – 71.5±5.5% and 59.9±6.6% ($P \leq 0.0001$; $n = 17$); 1 mM – 81.8±2.3% and 72.0±5.2% ($P \leq 0.0001$; $n = 25$); 10 mM – 100.0±3.7% and 100.0±4.9% of PI ($P \leq 0.0001$, $n = 12$; Figure 6.3), respectively. In contrast, METH was only toxic to CA3 sub-region at METH concentrations equal to and above 0.5 mM as follows (in mM): 0.5 – 42.4±5.4% ($P = 0.0003$; $n = 17$); 1 – 51.6±5.0% ($P \leq 0.0001$; $n = 25$); 10 – 100.0±7.5% of PI ($P \leq 0.0001$, $n = 12$; Figure 6.3). Given these results, we determined that the EC_{50} value for the three hippocampal sub-fields analyzed (0.5 mM METH), and we used this concentration in the following studies.

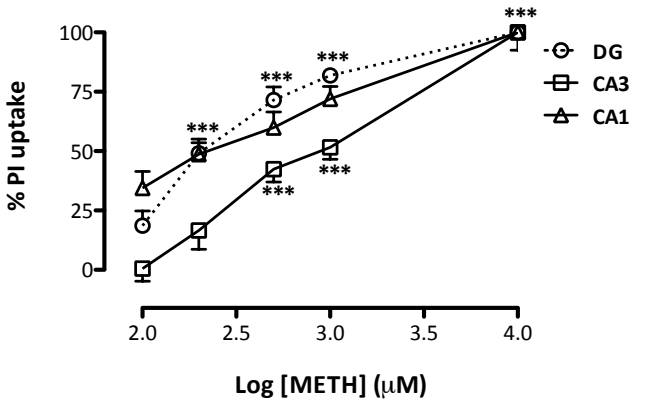


Figure 6.3. *METH induced cell death in mouse hippocampal cultures. Densitometry measurements of PI uptake induced by increasing concentration of METH (0.1 - 10 mM) in DG, CA3 and CA1 hippocampal sub-regions after 24h of METH exposure. The maximal PI uptake in each sub-region was set to 100%. Data are expressed as percentage of PI uptake (mean ± SEM), n = 13-41. $^{***}P < 0.001$ - Mann Whitney test, significantly different from the respective control (no drug exposure).*

6.4.2. NPY is neuroprotective against METH-induced neuronal cell death *via* Y_2 receptors

It is known that under toxic conditions NPY has an important neuroprotective role (Silva *et al.*, 2003b; Thiriet *et al.*, 2005; Xapelli *et al.*, 2007). Moreover, Thiriet and collaborators (2005) demonstrated that NPY protects striatal neurons from METH-induced cell death, mainly *via* Y_2 receptor. Despite these conclusions, to date there are no studies reporting the role played by the hippocampal NPY system under conditions of METH-induced cell death. To clarify this issue, we first demonstrated that mice hippocampal slice cultures express NPY and both Y_1 and Y_2 receptors by RT-PCR, using as a positive control the hippocampi of adult mice (Figure 6.4A). Then, we investigated if this peptide could prevent or attenuate neurotoxicity induced by METH (set to 100%), and we found that NPY reduced the PI uptake to values similar to the control in DG granule cell layer (Figure 6.4B), as well as in both CA3 (Figure 6.4C) and CA1 (Figure 6.4D) pyramidal cell layers as follows: 47.3±4.4% ($P \leq$

0.0001; $F_{(4,169)}$ = 26.97; *n* = 23-55), 59.3±5.9% ($P \leq 0.0001$; $F_{(4,185)}$ = 21.23; *n* = 23-56) and 50.8±4.9% of METH ($P \leq 0.001$; $F_{(4,191)}$ = 21.29; *n* = 23-57). To discriminate the receptor(s) responsible for the NPY-mediated neuroprotection, we tested the effect of Y_1 (1 μM BIBP3226) and Y_2 (1 μM BIIE0246) receptor antagonists. In all hippocampal sub-regions, we concluded that the blockade of Y_1 receptors has no effect in NPY-mediated neuroprotection (DG – 51.4±4.9%, Figure 6.4B; CA3 – 58.9±8.5%, Figure 6.4C; CA1 – 42.3±5.7% of METH, Figure 6.4B-D). However, the Y_2 receptor antagonist completely abolished the reduction of PI uptake induced by NPY, and this effect was similar throughout the three sub-field analyzed (DG - 94.6±7.6%; CA3 - 105.2±7.7% and CA1 - 81.4±7.47% of METH; Figures 6.4B-D). Representative fluorescent image of PI uptake (Figure 6.4E) shows low basal PI uptake in control slices, which was increased after METH treatment in all hippocampal sub-regions and returned to basal levels in the presence of 1 μM NPY. Moreover, the Y_2 receptor antagonist (1 μM BIIE0246) completely blocked the neuroprotective effect induced by NPY, as shown by the PI fluorescence similar to METH condition (Figure 6.4E).

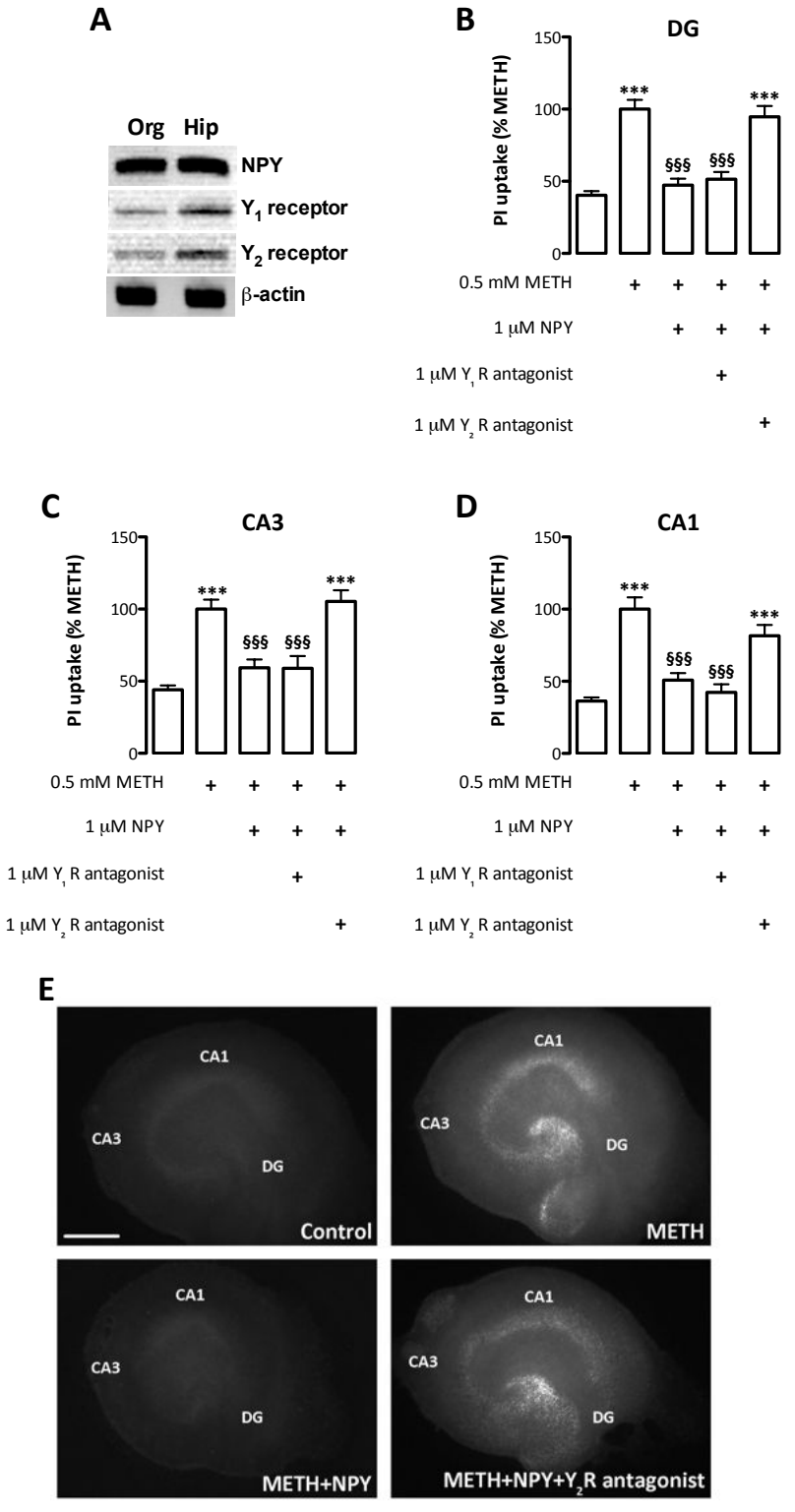


Figure 6.4. *NPY prevents METH-induced neuronal cell death in hippocampal slice cultures. (A) Naïve mouse hippocampal organotypic slice cultures express NPY as well as Y_1 and Y_2*

receptors. The mRNA levels were analyzed by RT-PCR and as a positive control we used the hippocampi of control adult mice (0.9% NaCl, i.p.). Legend: Org - Mouse hippocampal organotypic slice cultures; Hip - Adult mice hippocampi. (B-E) NPY (1 μM) inhibited the PI uptake induced by 0.5 mM METH in (B) DG granular cells, and in both (C) CA3 and (D) CA1 pyramidal cell layers. The protective effect of NPY was completely abolished in the presence of Y_2 receptor antagonist (1 μM BIIE0246), whereas Y_1 receptor antagonist (1 μM BIBP3226) had no effect. The PI uptake induced by 0.5 mM METH was set to 100%. The results are expressed as percentage of METH (mean ± SEM), n = 23-57. $^{***}P < 0.001$, significantly different from control (no drug exposure); $^{§§§}P < 0.001$, statitiscal significance when compared with 0.5 mM METH; using one way ANOVA following Bonferroni's test. (E) Representative fluorescence images of PI uptake in hippocampal organotypic slice cultures under several experimental conditions. Scale bars: 500 μm.

These results clearly show that the neuroprotective effect mediated by NPY against METH-induced neuronal cell death occurs *via* Y_2 receptor activation.

6.4.3. Selective activation of Y_1 or Y_2 receptors protects from METH-induced neuronal cell death

As previously described, the specific activation of NPY receptors, namely Y_1 and Y_2 subtypes, activates neuroprotective pathways that are able to protect hippocampal neurons from excitotoxic insults (Silva *et al.*, 2003b, 2007; Xapelli *et al.*, 2007, 2008). Thus, in the present study we also evaluated the specific role of Y_1 and Y_2 receptors against neuronal cell death induced by METH (0.5 mM). In DG and CA3 sub-regions, the activation of Y_1 receptors with 1 μM [Leu^{31},Pro^{34}]NPY partially prevented the increase of PI uptake induced by METH (DG – 63.4±6.2%, $P \leq 0.0001$, $F_{(3.152)} = 30.51$, n = 26-55, Figure 6.5A; CA3 – 71.9±7.9%, $P \leq 0.0001$, $F_{(3,160)} = 22.19$, n = 26-57, Figure 6.5B). However, the Y_2 receptor agonist (300 nM NPY13-36) completely prevented the cell death induced by METH (DG – 40.1±4.8%, Figure 6.5A; CA3 – 51.6±6.0%; Figure 6.5B). Regarding CA1 pyramidal cell layer, we verified that both Y_1 and Y_2 receptor

agonists were able to completely block neuronal cell death induced by 0.5 mM METH (57.6±6.9% and 43.6±4.7%, respectively; $P \leq 0.0001$; $F_{(3,157)} = 28.83$, n = 24-56; Figure 6.5C). The neuroprotection mediated by selective activation of Y_1 or Y_2 receptors are shown by representative fluorescent images in Figure 6.5D.

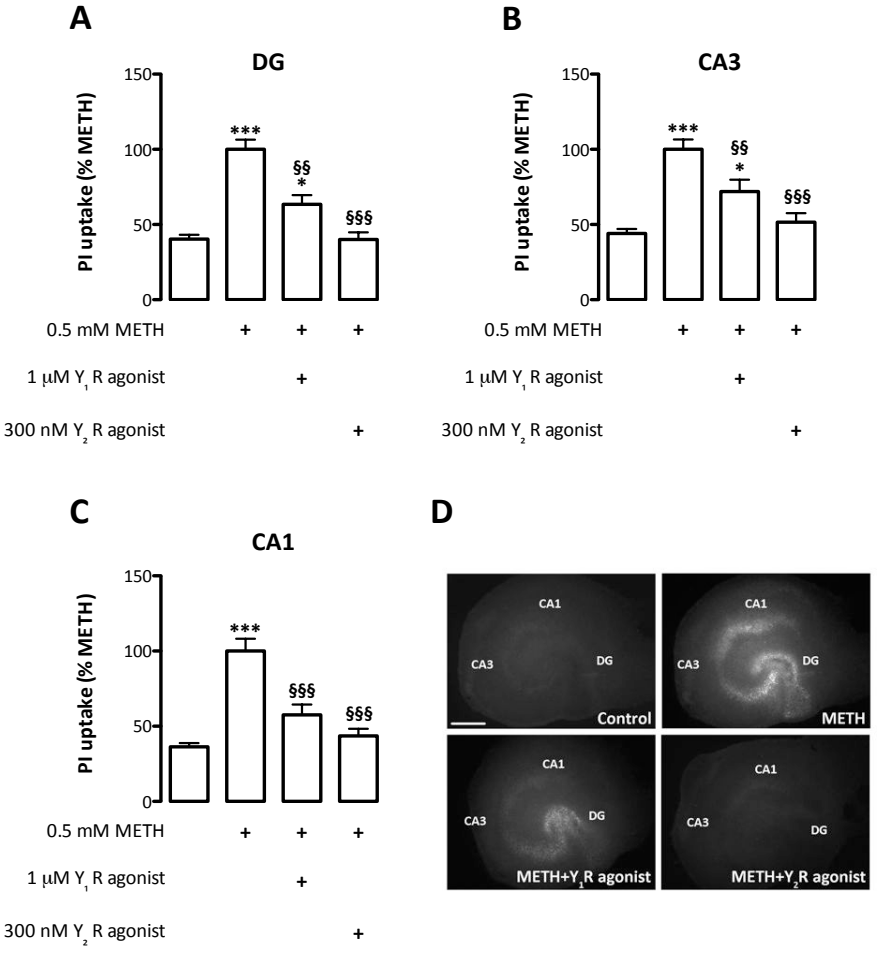


Figure 6.5. *Selective activation of Y_1 or Y_2 receptors has a protective effect against METH-induced hippocampal neuronal cell death. The Y_1 receptor agonist (1 μM [Leu^{31},Pro^{34}]NPY) partially prevented the cell death induced by 0.5 mM METH in (A) DG granular cell and (B) CA3 pyramidal cell layer, but completely prevented this toxic effect in the (C) CA1 pyramidal cell layer. Moreover, the specific activation of Y_2 receptors by 300 nM NPY13-36 complety blocked the METH toxicity in the (A-C) three hippocampal sub-regions analysed. The PI uptake induced by 0.5 mM METH was set to 100%. The results are expressed as percentage of METH (mean ± SEM), n = 26-57. $^{*}P < 0.05$, $^{***}P < 0.001$, significantly different from control (no drug exposure); $^{§§}P < 0.01$, $^{§§§}P < 0.001$,statitiscal significance when compared with 0.5*

mM METH; using one way ANOVA following Bonferroni's test. (D) Representative fluorescence microscopy images of PI uptake in hippocampal organotypic slice cultures under several experimental conditions. Scale bars: 500 μm.

In an attempt to better clarify protective role played by NPY receptors, we further examined the ratio between the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax, which is the major checkpoint for mammalian cell death pathway (Gross *et al.*, 1999). Hence, we verified that Bcl-2/Bax ratio was decreased following METH treatment (0.39±0.05, $P = 0.004$, $F_{(3,20)} = 11.88$, $n = 4\text{-}8$) when compared with control conditions (1.0±0.18; Figure 6.6). While the Y_1 receptor agonist (0.57±0.03, $P = 0.007$, $F_{(4,35)} = 18.83$, $n = 8\text{-}12$) did not interfere with the Bcl-2/Bax ratio observed in the presence of METH, the Y_2 receptor agonist increased this ratio that reached similar values to the control (1.1±0.17). Furthermore, the protective role of Y_2 receptors was blocked by the selective antagonist which down-regulated the Bcl-2/Bax ratio (0.22±0.10, $P = 0.0043$, $F_{(4,35)} = 18.83$, $n = 8\text{-}12$; Fig. 6.6). Taken together, these data suggest that Y_2 receptors have a central role in protection against neuronal cell death in DG, CA3 and CA1 sub-regions. Nevertheless, the specific activation of Y_1 receptors is also important in the protection of DG and CA3 cells, but seems to involve different pathways.

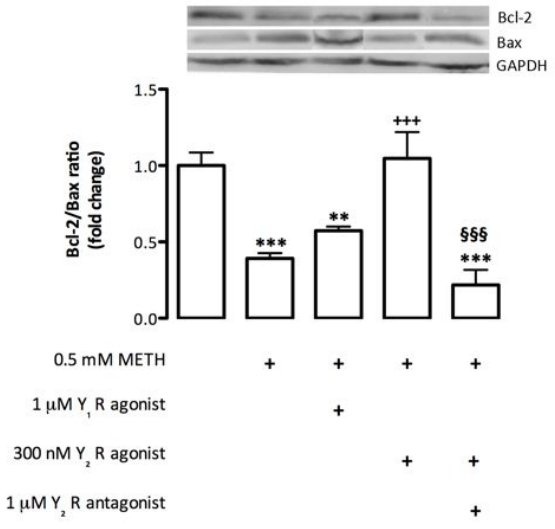


Figure 6.6. *METH (0.5 mM) decreased the Bcl-2/Bax ratio, which was prevented by the selective activation of Y_2 receptors (300 nM NPY13-36). When the Y_2 receptors were blocked (1 μM BIIE0246) the Bcl-2/Bax ratio returned to values similar to those observed in the presence of METH. Above the bars, representative Western blot images of Bcl-2 (26 kDa), Bax (21 kDa) and GADPH (34 kDa) are shown. The ratio in the presence of METH was set to 1. The results are expressed as fold change (value ± SEM), n = 4-6. $^{**}P < 0.01$, significantly different from control (no drug exposure); $^{§§}P < 0.01$, statitiscal significance when compared with 0.5 mM METH; $^{+++}P < 0.001$, statitiscal significance comparing with 0.5 mM METH plus 300 nM Y_2 receptor agonist; using one way ANOVA following Bonferroni's test.*

6.4.4. Y_2 receptors prevent METH-mediated microglial response and cell death

The activation of microglia is one of the hallmarks of METH-related neuropathology (Sekine *et al.*, 2008; Goncalves *et al.*, 2010; Silva *et al.*, 2010). In fact, we previously reported that METH intoxication induces microgliosis in the mouse hippocampus (Goncalves *et al.*, 2010). In the present study we aimed to investigate if METH could also affect microglial cells in the hippocampal organotypic cultures, and to disclose the role played by the Y_2 receptors in microglial activation. We concluded that METH triggered a microglial response

as shown in Fig. 6.7A. Accordingly, we verified that METH treatment leads to a significant up-regulation of CD11b fluorescent signal in the three hippocampal sub-regions analyzed as follows (control was set to 100%): DG - 451.8±49.2% ($P < 0.0001$; n = 49); CA3 - 401.3±38.8% ($P < 0.0001$; n = 60); CA1 - 560.3±57.0% ($P < 0.0001$; n = 42; Figure 6.7B). Furthermore, the Y_2 receptor agonist (300 nM NPY13-36) was able to prevent METH-induced increase of CD11b fluorescent signal (DG - 134.8±23.0%; $P < 0.0001$; n = 50; CA3 - 156.7±11.4%; $P < 0.0001$; n = 33; CA1 - 180.8±38.2%; $P < 0.0001$; n = 54; Figure 6.7A, B).

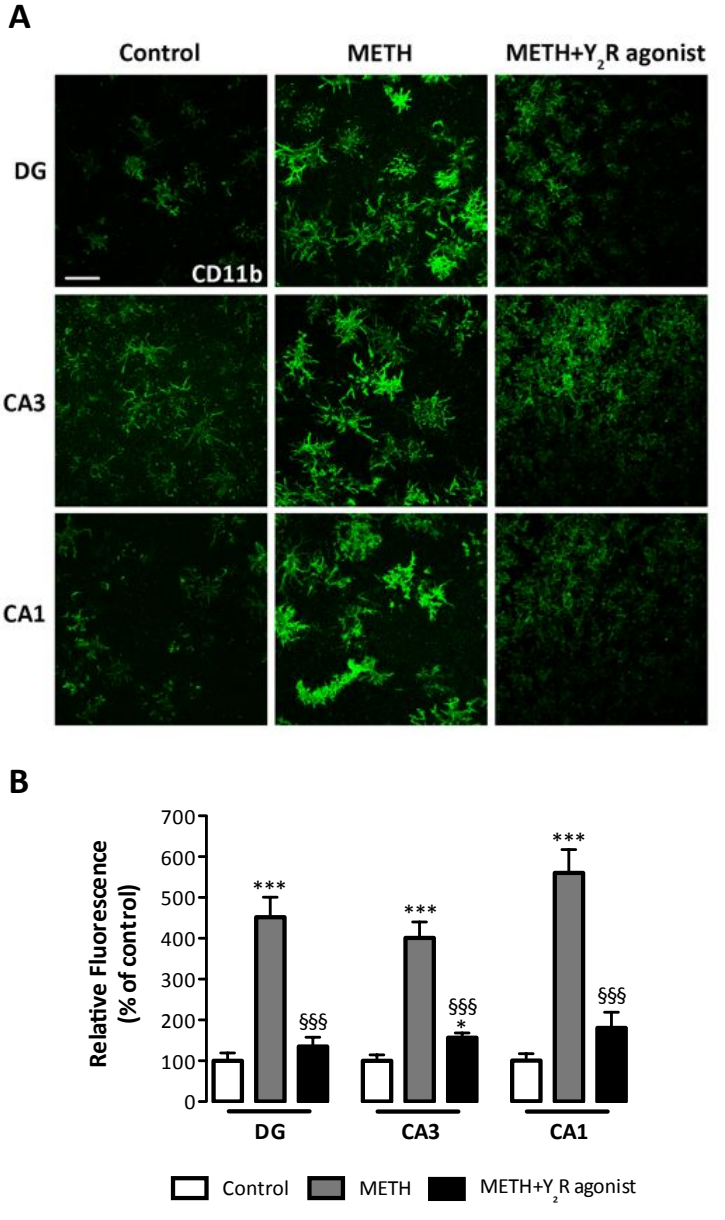


Figure 6.7. *Activation of microglial cells in mouse hippocampal organotypic cultures by METH. (A) Immunofluorescent labeling with anti-CD11b (green) and respective (B) fluorescence intensity quantification demonstrate that METH (0.5 mM) activates microglia in DG, CA3 and CA1 sub-regions. The Y_2 receptor agonist (300 nM NPY13-36) reduced METH-induced microglia activation in hippocampal formation. Data are expressed as percentage of control (mean ± SEM), n = 33-54. $^{*}P < 0.05$, $^{***}P < 0.001$, significantly different from control (no drug exposure); $^{+++}P < 0.001$, statistical significante comparing with 0.5 mM METH using one way ANOVA following Bonferroni's test. Scale bars: 50 µm.*

Since our results suggest a direct effect of METH on microglial cells and an important role of the NPY system, we hypothesized if NPY and its receptors could also protect against METH-induced microglial cell death. To answer this question, we used a mouse microglia cell line, which express NPY as well as both receptors (Ferreira *et al.*, 2010). The cell line was exposed to 1 mM METH (Coelho-Santos *et al*., 2012), which increased the number of apoptotic cells (26.0±1.5%, $P \leq 0.0001$, $F_{(4,148)} = 119.8$, n = 12-46) when compared with non-treated cells (1.1±0.2%; Figure 6.8). In accordance with organotypic hippocampal culture studies, NPY completely prevented microglial cell death induced by METH (3.9±0.2%; Figure 6.8A). Once again the Y_1 receptor is not involved in NPY-mediated protection (3.2±0.61%; Figure 6.8A), and the Y_2 receptor antagonist only partially blocked the effect of NPY (12.1±1.1%, $P \leq 0.0001$, $F_{(4,148)} = 119.8$, n = 12-46; Figure 6.8A). Then, we evaluated the specific contribution of each NPY receptor, and our results revealed that the activation of Y_1 receptors had no effect (22.5±2.2%; Figure 6.8B) in METH-mediated microglia cell death. Regarding Y_2 receptors, their activation completely protected microglial cells from METH toxicity (1.5±0.31%, $P \leq 0.0001$, $F_{(4,139)} = 107.8$, n = 13-46; Figure 6.8B), which was blocked in the presence of the selective Y_2 receptor antagonist (20.3±1.37%; Figure 6.8B). These data clearly demonstrate that the protection mediated by the NPY system against METH insult is relevant in different cell types, namely neurons and microglia.

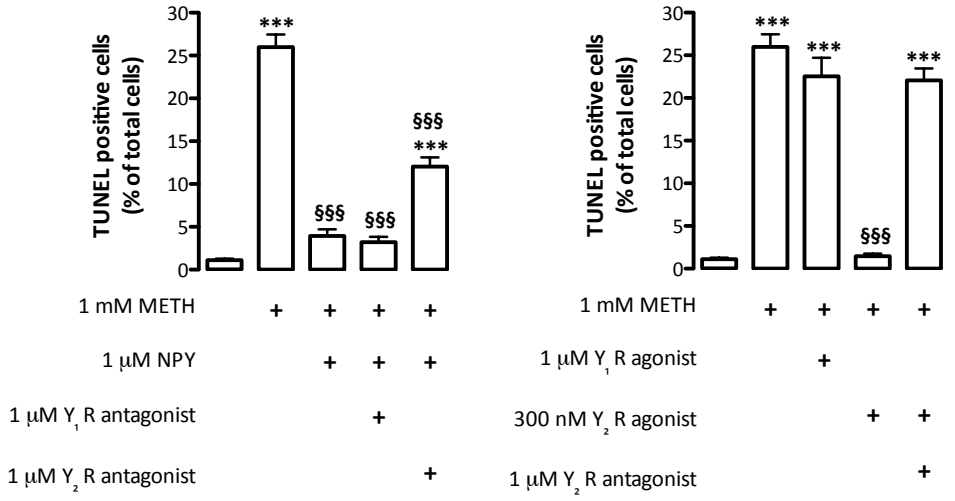


Figure 6.8. *Role of NPY, Y_1 and Y_2 receptors on microglial cell death induced by METH. (A) NPY decreases the toxic effect induced by 1 mM METH through, in part, Y_2 receptors. (B) The Y_1 receptor agonist did not induce protection against microglial degeneration induced by METH. However, the specific activation of Y_2 receptors (300 nM NPY13-36) completely prevented the increase of microglial cell death induced by 1 mM METH. The results are expresssed as percentage of total cells (value ± SEM), n = 15-46. $^{***}P < 0.001$, statistical significance when compared to control (no drug exposure); $^{§§§}P < 0.001$ statistical significance when compared to 1 mM METH; using one way ANOVA following Bonferroni's test.*

6.5. Discussion

In the present study, we aimed to explore the protective role of NPY system against METH-induced neuronal and microglial cell death, as well as microglial activation. The only available study regarding this issue was published by Thiriet and colleagues (2005) showing that METH administration (4x 10 mg/kg each 2h intervals; i.p.) increases and decreases NPY and Y_1 receptor mRNA levels, respectively, accompanied by a biphasic change of Y_2 receptor mRNA levels in the mouse striatum. Moreover, this study demonstrated that NPY reduces METH-induced striatal cell death, mainly *via*

activation of Y_2 receptors (Thiriet *et al.*, 2005). In fact, several lines of evidence suggest that NPY acts as an important neuroprotective agent (Silva *et al.*, 2005a; Xapelli *et al.*, 2006). Accordingly, in the present study we show that NPY is neuroprotective against METH-induced neuronal cell death in organotypic hippocampal slice cultures *via* Y_2 receptor activation, at least in part due to its ability to increase and decrease anti- and pro-apoptotic proteins levels, respectively. Furthermore, for the first time we report that the specific activation of Y_2 receptors is beneficial to microglia since it decreases microglial activation following METH exposure in hippocampal cultures, and also prevent microglial cell death induced by this drug.

Previously our group reported that, in hippocampal neurons, NPY through activation of both Y_1 and Y_2 receptors inhibits the intracellular Ca^{2+} concentration and glutamate release in response to depolarization (Silva *et al.*, 2001). Also, it was observed that 30 days after KA injection (10 mg/kg; i.p.) the mRNA expression levels of both NPY and Y_2 receptor were up-regulated in the rat hippocampus, suggesting an adaptive mechanism to control the hyperexcitability of the epileptogenic process (Silva *et al.*, 2005b). Here, we also report that NPY has an important neuroprotective role against METH-induced neuronal cell death through Y_2 receptors. Moreover, this effect was observed in the DG granular cell layer, CA3 and CA1 pyramidal cell layers. Importantly, NPY receptors are differently distributed within the hippocampal formation. In fact, high levels of Y_1 receptors were described in the granular cell layer of the DG, while moderate levels are expressed in CA3 and CA3 pyramidal cells (Caberlotto *et al.*, 1997; Gackenheimer *et al.*, 2001). Regarding Y_2 receptors, very high levels are found in CA3 and CA1 sub-fields (Caberlotto *et al.*, 1998; Gackenheimer *et al.*, 2001). Furthermore, the localization of these receptors in the nerve terminals is also distinct since Y_2 receptors are mostly presynaptic, while the Y_1 receptor subtype is present in both at pre- and post-synaptic terminals (Silva *et al.*, 2001). So, we wondered In fact, several authors showed that the distribution of each NPY receptor is different within hippocampal formation. So,

we wondered if the role of Y_1 and Y_2 receptors were similar within the hippocampus under METH toxicity. Our results show that the specific activation of Y_1 receptor only partially prevented METH toxicity in DG and CA3 sub-regions, while the Y_2 receptor activation was able to confer full protection. Regarding CA1 pyramidal cells, the selective activation of both receptors had a complete neuroprotective effect against METH-induced neuronal cell death. Indeed, the identification of the selective NPY receptor(s) involved in physiological and pathological conditions is of extreme importance since the role played by each one can be very different. Previously, our group showed that, in rat hippocampal organotypic cultures, the activation of both Y_1 and Y_2 receptors completely protect DG granular cells or CA3 pyramidal cells from neurodegeneration induced by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or KA, respectively, but only Y_2 selective agonist was able to prevent CA1 pyramidal cells from both insults (Silva *et al.*, 2003b). However, in mouse organotypic slice cultures exposed to AMPA, only the Y_2 receptors exerted a neuroprotective effect in CA3 and CA1 sub-fields. (Xapelli *et al.*, 2008). In other study, we also verified that specific Y_1 and Y_2 receptor activation 30 min post-KA insult prevent cell degeneration in all three sub-regions analyzed (Xapelli *et al.*, 2007). Moreover, it was demonstrated that this time-point is crucial for NPY, *via* Y_2 receptor activation, rescue rat hippocampal neurons after intra-hippocampal KA injection (Smialowska *et al.*, 2009). The same study revealed that Y_2 receptors significantly reduced the infarct volume produced by a middle cerebral artery occlusion (MCAO) in rats (Smialowska *et al.*, 2009). In agreement, our data highlights the role of Y_2 receptor subtype as the main responsible for NPY-mediated neuroprotection against METH toxicity. However, this receptor is also involved in hippocampal memory processing and its activation can lead to deficits in memory formation because of glutamate release attenuation and, consequently, long-term potentiation (LTP) (Sorensen *et al.*, 2008a; Sorensen *et al.*, 2008b). These observations suggest that the role played by the NPY receptors is dependent of several factors, such as the type of insult and brain region affected.

In an attempt to better clarify the neuroprotective role of NPY receptors, we also showed that the Y_2 receptors activation up- and down-regulated the protein levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax, respectively. Indeed, the cellular surveillance is dependent on a molecular balance that regulates the ratio of anti- and pro-apoptotic proteins (Kluck *et al.*, 1997; Gross *et al.*, 1999). Actually, it was demonstrated that METH (40 mg/kg; i.p.) led to an increase and decrease levels of Bax and Bcl-2, respectively, in mouse olfactory bulb at 4h post-injection (Deng *et al.*, 2007). Furthermore, the same occurred after repeated METH treatment (4 x 5 mg/kg; i.p.) in mitochondrial fractions of rat pre-frontal cortices (Bachmann *et al.*, 2009). Also, some studies showed the involvement of Y_2 receptor activation in extracellular signal-regulated kinase (ERK) phosphorylation (Mullins *et al.*, 2002; Decressac *et al.*, 2011). In fact, the stimulation of Y_2 in a mice model of Parkinson's disease triggered the activation of ERK1/2 signaling pathway, which was related with neuroprotective effect of NPY against 6-hydroxydopamine (6-OHDA)-induced toxicity on nigral dopaminergic cell bodies and striatal terminals (Decressac *et al.*, 2011). Moreover, several authors reported that the phosphorylation of ERK, and consequently translocation to the nucleus, leads to transcription of anti-apoptotic genes, such as Bcl-2 (Almeida *et al.*, 2005; Huang *et al.*, 2010; Kitazawa *et al.*, 2010). On the other hand, Y1 receptor activation did not interfere with METH-induced down-regulation of Bcl-2/Bax ratio, which suggests that in the present experimental condition the Y1 and Y2 receptors trigger different signaling pathways. In fact, as above-mentioned, previous studies demonstrated differences between both receptor actions. Specifically, hippocampal Y1 receptors have been suggested to mediate seizure-promoting effect, while seizure suppression by NPY is predominantly mediated by Y2 receptors (Woldbye et al., 2010; Olesen et al., 2012). Moreover, it was previously described that in cultured rat hippocampal neurons, Y1 receptor activation inhibits Ca2+ currents mainly through N-type voltage-gated calcium channels (McQuiston et al., 1996; Silva et al., 2003b), while Y2 receptors inhibit N-, P-/Q- and L-type calcium channels (Qian et al., 1997; Silva et al., 2003b).

Nevertheless, previous studies clearly showed that both receptors could be involved in neuroprotection. Importantly, this seems to be an effect mediated mainly by stimulation of Y2 receptors and, to a lesser extent, of Y1 receptors (Silva et al., 2003a; Thiriet et al., 2005; Xapelli et al., 2007), which is in accordance with our data. Here, we can also conclude that the protection afforded by Y1 signaling against METH-induced neuronal death in hippocampal slice cultures did not involve the regulation of Bcl-2/Bax proteins.

Besides neurons, glial cells are also affected by METH (LaVoie *et al.*, 2004; Goncalves *et al.*, 2010; Kitamura *et al.*, 2010). We have previously shown that METH intoxication originated hippocampal gliosis, which remained until at least 7 days after drug administration (Gonçalves *et al*., 2010). Microglia express several neurotransmitters, including neuropeptides, that are involved in the control of its functions (Pocock & Kettenmann, 2007). However, only recently it was described that NPY is present in retina (Álvaro et al., 2007) and brain microglia (Ferreira et al., 2011). Additionally, Ferreira and collaborators (2011) demonstrated that brain microglia exhibit Y1 and Y2 receptors in both surveying (previously described as resting microglia) and reactive state (Hanisch and Kettenmann, 2007). Although the anti-inflammatory effect of NPY has been already reported (Bedoui *et al.*, 2003; Nave *et al.*, 2004; Bedoui *et al.*, 2007; Bedoui *et al.*, 2008; Mitic *et al.*, 2011), only few studies have explored the role of NPY system in other microglia functions. Very recently, our group found that, upon lipopolysaccharide (LPS) challenge, NPY through activation of Y_1 receptors inhibits NO and interleukin-1β (IL-1β) production, as well as motility of microglial cells (Ferreira *et al.*, 2010; Ferreira *et al.*, 2011)). Here, we demonstrate that Y_2 selective activation decreases microglia activation, which is one of the features of METH-induced neuropathology (Goncalves *et al.*, 2010; Kitamura *et al.*, 2010). Additionally, we identified that NPY, mainly *via* Y_2 receptors, is a protective agent against METH-induced microglia cell death. Thus, our work corroborates the findings showing that anti-inflammatory role of NPY is predominantly mediated by Y_2 receptor signaling (Nave *et al.*, 2004;

Mitic *et al.*, 2011). In fact, Nave *et al*. (2004) showed that Y_2 receptor activation ameliorates the LPS-induced leuko-, granulo- and lymphopenia. Furthermore, more recently it was described that leucocyte infiltration following LPS administration was suppressed by NPY *via* both Y_1 and Y_2 receptors (Mitić *et al.*, 2011).

In conclusion, the present study reports that NPY affords protection against METH-induced neuronal and microglial toxicity *via* Y_2 receptors, which allow us to suggest this NPY receptor subtype as promising tool to prevent cell death induced by METH.

Chapter 1

General Conclusions

GENERAL CONCLUSIONS

In the present thesis, we concentrated our work on two main issues: 1. The involvement of neuroinflammation in METH-induced neuropathology; 2. The neuromodulatory role of NPY system in the hippocampus under METH conditions .

7.1. The involvement of neuroinflammation in METH-induced neuropathology

We were particularly interested in clarifying the role of neuroinflammatory processes as mediators of METH-induced neuropathology in the mice brain, especially in the hippocampus. Thus, in order to clarify whether an acute high dose of METH triggers a neuroinflammatory response, we started by evaluating the possible alterations of IL-1β, IL-6 and TNF-α mRNA levels in the mice hippocampus, frontal cortex and striatum. We observed that METH increased the IL-6 mRNA levels in the three brain regions analyzed, and TNF-α mRNA levels in the hippocampus and frontal cortex. However, no changes in IL-1β mRNA levels were detected. Moreover, since the hippocampus seems to be very susceptible to METH and exhibits an early up-regulation of TNF-α mRNA expression levels post drug treatment, we decided to explore the effect of an acute METH administration on mice hippocampal glial cells and neurons, as well as to analyze the changes in protein levels of TNF system. We verified that METH leads to a neuroinflammatory response in the mice hippocampus characterized by astrogliosis, microglial activation and augment of protein levels of TNF-α and its receptor TNFR1. Furthermore, we also detected that METH induces neuronal dysfunction with alterations in the cytoskeleton, synaptic and axonal proteins. Despite of these changes in glial and neuronal populations, there was no evidence of cell death in the hippocampus. In addition, we demonstrated that the pre-treatment with an anti-inflammatory

drug (indomethacin) was able to prevent the toxic effects of METH in the mice hippocampus. Taking in consideration that the TNF-α system is altered by METH, we further investigated its role under conditions of METH-induced microglial toxicity. It was demonstrated that endogenous TNF-α *per se* did not affect METH-induced cell death, which suggests that alterations on this system are a consequence rather than a cause of METH-induced microglial apoptosis. On the other hand, exogenous low concentrations of TNF-α prevented microglial and neuronal toxicity induced by METH.

7.2. The neuromodulatory role of NPY system in the hippocampus under METH conditions

NPY is involved in several brain functions, and has been shown to be an important neuroprotective agent. In the present work, we found that METH is able to increase the NPY levels, as well as to induce significant alterations in levels and activity of its receptors, namely the Y_2 receptor subtype. Moreover, we described that the METH-induced memory deficits are mediated by Y_2 receptor signaling, which involved the disruption of AKT/mTOR signaling pathway. Overall, we demonstrated the involvement of NPY in cognitive impairment mediated by METH, indicating that this neuropeptide has a detrimental role in mice hippocampus under METH intoxication. Importantly, the METH administration schedule that we used did not induced cell death. So, it seems that NPY role depends on several aspects, including the nature and severity of the insult. In fact, under conditions of cell death NPY seems to have a clear protective effect. Thus, we further evaluated the role of NPY and its receptors against METH-induced hippocampal neuronal cell death and microglial activation. Our results showed that NPY *via* Y_1 and Y_2 receptors is protective. Nevertheless, the Y_2 receptor subtype assumes a predominant role in this neuroprotection, in part due to its ability to up- and down-regulate the protein levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax. Similar to *in vivo*

studies, METH caused microglial activation, which was prevented by the blockade of Y_2 receptors.

Overall, our data revealed that neuroinflammation is an important feature of METH-induced brain dysfunction, and that NPY, specially the Y_2 receptor subtype, is a target to take into consideration in future attempts to treat brain pathologies associated with METH consumption.

Chapter 8

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