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Effect of adenosine receptors on 3, 4 methylene dioxy methamphetamine induced hyperthermic, neuroinflammatory and neurotoxic effects in mouse brain

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Dedicated to my beloved parents and my Fiancée

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-Amit Khairnar

List of abbreviations

MDMA	3, 4 methylenedioxymethamphetamine
MDA	3,4 methylenedioxyamphetamine
METH	Methamphetamine
DA	Dopamine
NE	Norepinephrine
BBB	blood brain barrier
ADE	adenosine deaminase
iNOS	inducible nitric oxide synthase
AKA	adenosine kinase
NO	nitric oxide
COX-2	cyclooxygenase 2
ERK	extracellular signal-regulated kinase
TH	tyrosine hydroxylase
GABA	gamma-amino butyric acid
5-HT	5-hydroxytryptamine
SERT	serotonin transporter
VMAT-2	vesicular monoamine transporter 2
5-HIAAC	5-hydroxyl indole acetic acid
MS	multiple sclerosis
TNF α	tumour necrosis factor α
PGE_2	prostaglandin E_2
IFN γ	interferon γ
GLT	glutamate transporters
NMDA	N-methyl-D-aspartic acid

GPCRs	G-protein-coupled-receptors
CNS	central nervous system
GFAP	glial fibrillary acidic protein
DHBA	dihydroxybenzoic acid
SNc	substantia nigra pars compacta
HHMA	3, 4 –dihydroxymethamphetamine
I.C.V	Intracerebroventricular
ETC	electron transport chain
HHA	Dihydroxyamphetamine
ROS	reactive oxygen species
MAO	monoamine oxidase
COMT	catechol-O-methyl transferase
HMA	3-methoxy,4-hydroxyamphetamine
SULT	Sulfotransferase
ALS	amyotrophic lateral sclerosis
UDPGT	Glucuronosyltransferase

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1. Introduction

1.1. History of MDMA

3, 4 Methylenedioxymethamphetamine (MDMA) was first synthesized and patented by the German pharmaceutical company Merck in Darmstadt around 1912, merely as a precursor in a new chemical pathway which was patented in order to avoid an infringement of existing patent for the synthesis of the clotting agent hydrastinine (Freudenmann RW *et al*., 2006). The first formal animal study on MDMA done by Hardman HF et al. in 1953-54 (US army studies) consisted of a number of LD50 determinations on five laboratory animal species, including the mouse. MDMA was first used by humans in the late 1960s, where it showed its properties of inducing feelings of well-being and increased communication (Watson L and Beck J. 1991). There was a phase of therapeutic enthusiasm for this drug as adjunct to psychotherapy due to its ability to enhance feelings of openness and trust and cause a sense of deep harmony in the self and in relationships with other persons (Grinspoon L and Bakalar JB. 1986). The drug was introduced in clinical psychotherapeutic practice on the West Coast of the United States in the beginning of 1976 (Shulgin A. 1990). There were reports of cases of toxicity and deaths from exposure to large doses of MDMA in United States and Europe (Dowling GP et al., 1987) and several researchers reported long-term neurotoxic effects of MDMA in laboratory animals (Ricaurte G *et al*., 1985; Schmidt CJ *et al*., 1986; Stone DM *et al*., 1986). Until the mid 1980s, drug use was restricted predominantly to people taking the drug when alone in a small party (Peroutka SJ *et al*., 1988). Whereas since the late 1980s, MDMA has been used as a street drug (street names include “Ecstasy”, “XTC”, “Adam”, “Essence”, “Clarity”). The Food and Drug Administration placed the compound on Schedule I control substance on July1, 1985. MDMA is now used extensively at dance clubs at parties called “raves”, and it is this new use that has probably given rise to a substantial increase in the number of reports of toxic reactions and deaths (Schwartz RH and Miller NS. 1997). Despite increasing reports of the

potential neurotoxicity, as well as deaths, related to MDMA use, the popularity of this drug has increased tremendously over the years making it at high concern for mental health professionals.

Table 1: Milestones from the history of MDMA/Ecstasy (Freudenmann RW *et al*., 2006)

Year	*Event*
1912	First synthesis of MDMA by Köllisch at Merck (Darmstadt, Germany), secured by German patent 274350
1927	First pharmacological tests with MDMA by Oberlin at Merck
1952	Basic toxicological tests with MDMA by van Schoor at Merck
1953/4	First formal animal study in five species using MDMA and seven other psychotropic drugs (University of Michigan); secret, US army-sponsored study, unpublished until 1973
1959	Re-synthesis of MDMA by Fruhstorfer at Merck
1960	First regular scientific paper on MDMA (in Polish) describing an MDMA synthesis
1970	First detection of MDMA in tablets seized in the streets of Chicago
1978	First MDMA studies in humans by Shulgin and coworkers reporting on chemistry, dosage, kinetics and psychotropic effects
1984	MDMA's street name 'ecstasy' was coined in California
1985–8	MDMA became a Schedule I controlled substance in the United States and banned in most others soon thereafter

1.2. Epidemiology and abuse studies

The national institute on drug abuse in 2008 estimated 2.1 million Americans aged 12 and older had abused MDMA at least once in their life. Over 32 million people or almost 10% of the adult population in the European Union and Norway used the drug in 2008, according to the annual report of the European Monitoring Center for Drugs and Drug Addiction (EMCDDA). In that around 2 million drug users in Europe preferred amphetamine while ecstasy was used by 2.5 million people. Approximately 12 million have tried amphetamine and 10 million have tried ecstasy at least once in their lives. The use of ecstasy is overtaking the other amphetamines and getting the second place, after cannabis, in both general population and school surveys (EMCDDA 2008). It has been estimated that the percentage of ecstasy consumers in Italy is stable and approximately represent 22% of total drug abusers (*http://aloearborescens.tripod.com/fumo.htm*).

1.3. Statistics showing emergency room visits due to drug abuse in USA

From the data available in literature very clear picture of consumption of MDMA can be seen. First graph shows that total rate of Emergency Room (ER) visits among 'ecstasy' users is approximately 1 in 600 users per year. The second graph shows the increase in ER visits from 1994 to 2002 with MDMA abuse. The number of ER visits rapidly increased from 1994-2001, declining in 2002 (apparently due to reduced rates of use, Fig. 2). Most of this increase was simply due to the greatly increased number of people using 'ecstasy' (http://www.thedea.org/statistics.html).

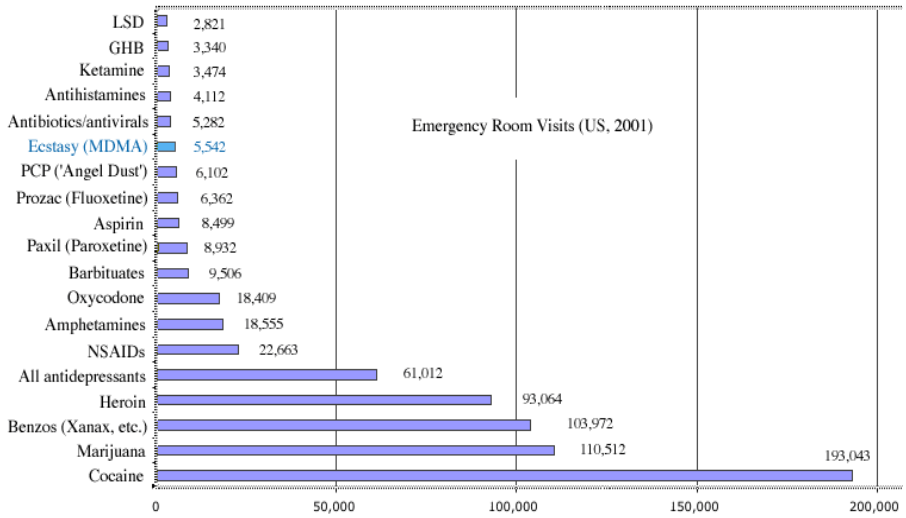

Figure 1: Emergency room (ER) visits due to drug abuse in 2001 (USA).

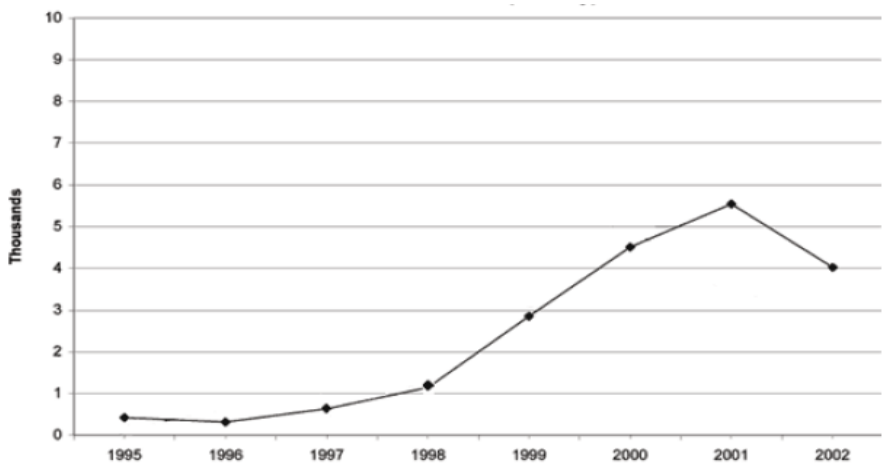

Figure 2: Graph showing emergency room (ER) visits due to MDMA drug abuse from 1995 to 2002 (USA).

Table 2: Deaths caused by MDMA abuse from 1994 to 2001 (USA).

Year:	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**	**2000**	**2001***
Reported Deaths:	**1**	**6**	**8**	**3**	**9**	**42**	**63**	**76**

In the Table 2 it is shown the number of deaths due to MDMA abuse. The increase in death reports (Table 2) does, however, better match the increases in Emergency Room visits (Figure 2). The basis of reporting MDMA deaths is not exactly associated with MDMA abuse as ecstasy tablets usually contains varying amounts of MDMA and may contain some other substances such as caffeine, amphetamines and 3,4 methylenedioxyamphetamine (MDA) which could be harmful to humans. Administration of MDMA has reinforcing effects as showed by studies on MDMA – induced place preference (Bilsky EJ et al., 1990) and intravenous self administration in rodents (De La Garza R et al., 2007). Moreover, the reinforcing effects of MDMA have been investigated in rhesus monkeys, although its reinforcing efficacy appears to be less than that of cocaine or methamphetamine (Lile JA et al., 2005).

1.4. Chemical Structure and Mechanisms of Action of MDMA

As its chemical name implies, MDMA bears the intrinsic structure of amphetamine (AMPH) with an *N*-methyl group and a methylenedioxy-ring substitution on the third and fourth carbon of the phenyl ring (Figure 3). It is thought that this variation in structure is responsible for dichotomous effects on brain neurochemistry exerted by these two substances. More specifically, AMPH and its derivative, methamphetamine (METH) has potent and long lasting effects on the dopamine (DA) neuromodulatory system while, in the long-term, MDMA affects mainly the serotonergic system. Additionally, MDMA more subtly affects the dopamine, norepinephrine (NE), gamma-aminobutyric acid (GABA), glutamate, and other systems as well (Green AR *et al*., 2003).

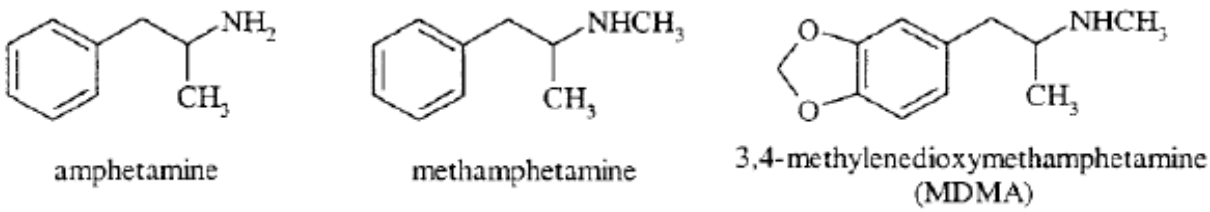

Figure 3: Chemical structure of amphetamine, methamphetamine, and MDMA (Green AR *et al*., 2003).

Acutely, MDMA causes a rapid efflux of serotonin (5-hydroxytryptamine; 5-HT), NE, and DA from respective monoaminergic terminals. In terms of serotonergic release, this effect is mediated by the drug's interaction with both the plasmalemmal serotonin transporter (SERT) as well as with the intracellular vesicular monoamine transporter 2 (VMAT-2), both proteins being involved in the selective transport of 5-HT across phospholipid bilayers. More specifically, MDMA is a substrate for both SERT and VMAT-2, allowing it to enter the terminal and subsequently vesicles bearing 5-HT, respectively (Rudnick G and Wall SC. 1992). Once inside the vesicles, the slightly alkaline nature of MDMA causes dissipation of the proton gradient between the vesicle and the cytosol necessary for proper functioning of VMAT-2, and in this respect, it inhibits VMAT-2-mediated influx and proper storage of 5-HT in the terminal (Sulzer D and Rayport S. 1990). Coupled with its ability to cause functional reversal of both VMAT-2 and SERT, MDMA allows 5-HT to passively efflux from terminal vesicles and subsequently from the neuron itself, ultimately leading to a global increase in extracellular 5-HT throughout brain regions bearing raphe afferents (Rudnick G and Wall SC. 1992). This effect is further potentiated by MDMA-induced inhibition of 5-HT reuptake, as consequence of competition for SERT-binding by both 5- HT and MDMA.

1.5. Pharmacology and Toxicity of MDMA

1.5.1. In Humans

MDMA the main component of ecstasy tablet has various harmful health effects in humans. The neurotoxic dose of MDMA in non-human primates approaches the dose of MDMA typically taken by recreational MDMA users and it is found to be 1.5 to 1.7 mg/kg in humans and 5 mg/kg in monkeys (www.drugtext.org/library/articles/ricaurte.htm). The acute effects after taking MDMA are hyponatraemia (headaque, confusion or altered mental state, seizures), hyperthermia is one of the causes for death due to MDMA. Approximately 15 young persons die every year from acute MDMA toxic effects. The reason may be that MDMA is usually taken recreationally in dance clubs or in rave parties in hot, crowded rooms (both conditions leads to larger elevation of MDMA-induced body temperature in animals) therefore clinically such conditions could increase the possibility of subsequent cerebral neurotoxic effect. Several case reports have been reported fatal hyperthermia after ingestion of ecstasy. The patient collapsed with the seizures they tended to have a very fast heart rate and very low blood pressure and body temperature as high as 43℃ (Henry JA et al., 1992; Green *et al*., 2004). Chronic studies by Mc Cann *et al*., 1994 found selective reductions in cerebrospinal fluid of 5 – hydroxyindoleacetic acid (5-HIAA) in MDMA users compared to control that never used it. Positron emission tomography (PET) studies showed evidence of decreased 5HT transporter sites correlated with the degree of MDMA exposure. It has also been found that, there is altered neuroendocrine function in MDMA users which can be correlated to alterations of hypothalamic 5HT function, suggestive of MDMA-induced 5-HT neurotoxicity. Neuropsychiatric testing methods found that MDMA users, compared to controls, had deficits in verbal and visual memory (Morgan MJ. 1999). Pharmacological depletion of brain 5-HT typically leads to dramatic decrease in non-rapid eye movement (NERM) sleep with less dramatic decreases in rapid eye movement (REM) sleep associated decreased total sleep time compared to controls (Mc Cann UD et al., 2000).

1.5.2. In Monkeys

Monkeys administered with MDMA showed a marked reduction in the number and density of 5-HT containing nerve fibers in the cortical, but also in the sub cortical regions. The MDMA treated animal has much fewer 5-HT containing axons. A series of studies were recently completed where monkeys were examined seven years after MDMA treatment. These animals still showed evidence of serotonin axon loss. This suggests that in monkeys the toxic effect of MDMA may be permanent (www.drugtext.org/library/articles/ricaurte.htm). The available preclinical data from monkeys tend to suggest that one or two episodes of repeated, high-dose exposure to MDMA are not sufficient to produce obvious disruptions of cognitive or behavioral function, despite producing large and lasting depletions of 5-HT in the neocortex (Frederick DL *et al*. 1998; Taffe MA *et al*. 2001; Winsauer PJ *et al*., 2002). MDMA produces an acute hyperthermia in unrestrained rhesus monkeys, much as it does with rats, mice, pigs, rabbits and humans (Taffe MA *et al*., 2006).

1.5.3. In Rats

MDMA administration in rats is known to release 5-HT in striatum and medial prefrontal cortex dose dependently which may lead to marked decrease in 5-HT concentration (Green AR *et al*., 2003). Treatement with MDMA also decreases 5-HT transporter level. A significant reduction in tryptophan hydroxylase (TPH), a rate limiting enzyme required for 5-HT synthesis is observed in the hippocampus, striatum and frontal cortex of hyperthermic animal's resulting in decreased cerebral tissue concentrations of 5-HT and 5-HIAA after MDMA administration. However tryptophan hydroxylase (TPH) activity was unaltered in hypothermic animals after administration of MDMA (Stone DM *et al*., 1987, Che S *et al*., 1995). In addition MDMA also inhibits the catabolic enzyme monoamine oxidase (MAO) having potency 10 times greater at MAO–A than MAO-B. MDMA also rapidly increases dopamine release in striatum and causes a sustained depletion of DOPAC and HVA. The release of dopamine by MDMA occurs through entering in dopamine nerve terminals and is modulated by 5-$HT_{2A/2C}$ receptors. MDMA administration to rats

has generally been reported to produce a marked hyperthermic response of approximately +1-2℃ when kept in 22℃ ambient temperature, while hypothermic response at 17℃ indicating a high sensitivity to small changes in ambient temperature. MDMA also induces increase in glial fibrillary acidic protein (GFAP) expression in the hippocampus that paralleled 5-HT damage and was prevented in the same way by α- lipoic acid administration (Green AR *et al*., 2003).

1.5.4. In Mice

In contrast to the MDMA pharmacological effects in rats, MDMA administration in mice causes a small decrease in 5-HT and 5-HIAA in cortex and hippocampus with little effect on striatum. Whereas it causes a rapid release of dopamine in striatum and reduces striatal content of both dopamine and its metabolites HVA and DOPAC. It is observed that administration of dopamine reuptake inhibitor GBR 12909 enhanced the MDMA induced increase in extracellular dopamine concentration indicating that MDMA may enter nerve terminal by diffusion and not via dopamine reuptake site (Camarero J et al., 2002). It has also been shown that proinflammatory molecules such as inducible nitric oxide are increased one day after administration of MDMA and that their inhibition provides protection against MDMA-induced loss of DA in striatum (Granado N et al., 2008). A study by Thomas DM et al., 2004 showed that MDMA induces significant microgliosis in striatum and SNc. MDMA induced hyperthermia though much more variable has been seen in mice. Also in mice MDMA at a dose of 10mg/kg induced hypothermia while 30mg/kg induced hyperthermia followed by hypothermia in Swiss Webster mice. The locomotor activity seen in mice is found to be mediated by 5-HT_{1B} receptor (Green AR et al., 2003).

1.6. Mechanism Involved in MDMA Toxicity

Though lot of studies have been carried out to understand mechanism involved in MDMA induced neurotoxicity still there is no much success. It is very well known that free radicals are involved in MDMA induced neurotoxicity but it is not yet known the source for free radicals which

may be from release of excess of dopamine, from mitochondrial complex I inhibition, or from formation of toxic MDMA metabolites.

1.6.1. Oxidative stress

Several studies using animal models supported the involvement of oxidative stress in MDMA neurotoxicity. The role of oxidative stress is further supported by the findings that neurotoxic effects of MDMA can be attenuated by free radical scavengers and anti-oxidants. The reactive oxygen and nitrogen species involved in oxidative stress are suppose to be formed from release of excess of dopamine in cytosol or from formation of neurotoxic MDMA metabolites (Puerta E *et al*., 2010). MDMA induces rapid and powerful release of dopamine in mice which is metabolised by MAO-B and leads to formation of DA quinones as well as hydrogen peroxide. Attenuation of DA release by lesioning DA neurons or blocking DA transporter has been shown to protect against the long term toxicity of MDMA. In addition, oxidation of 5-HT, MDMA itself, and thioether metabolites of MDMA has also been implicated in MDMA neurotoxicity. MDMA has now been shown to increase hydroxyl radical formation in rats; consequently these highly reactive free radicals can lead to the generation of lipid peroxidation and oxidize proteins in the nerve terminals (Quinton MS et al., 2006). Camarero J et al., (2002) reported that administration of MDMA led to rise in the formation of 2, 3 dihydroxybenzoic acid (2, 3 DHBA) and malonyldialdehyde, a lipid peroxidation product, in mice striatum. In addition to reactive oxygen species, reactive nitrogen species now appear to play a major role in mediating MDMA-induced neurotoxicity. Neuronal nitric oxide synthase activation seems to be involved in MDMA neurotoxicity which generates NO. This generated NO and peroxide radical from DA metabolism combines together to form peroxinitrite ($ONOO^-$) which promotes autooxidation of DA to DA quinone and also has been found to inhibit DAT (Chipana C et al. 2006). Similarly, Colado MI et al reported in 2001 that neuronal NOS inhibitors provided significant neuroprotection against MDMA – induced long term dopamine depletion in mice.

The development of various transgenic knockout mouse models has helped to elucidate various mechanisms underlying MDMA-mediated neurotoxicity. Homozygous and heterozygous copper/zinc superoxide dismutase (an antioxidant enzyme) transgenic mice were partially protected against MDMA-mediated DA damage. Moreover, MDMA causes a decrease in catalase and glutathione peroxidase, and an increase in lipid peroxidation in wild-type animals, effects that are not observed in the homozygous mice (Jayanthi S *et al*., 1999).

1.6.2. Excitotoxicity

Excitotoxicity includes succession of several events, excessive glutamate release, activation of glutamate receptors and increase in intracellular calcium levels which leads to generation of free radicals and nitric oxide (Bruno V. et al., 1993; Yamamato BK et al., 2010). In fact glutamate and other excitatory amino acids have been linked to several neurodegenerative disorders suggesting a possible role of glutamate in Methamphetamine and MDMA- induced terminal degeneration (Lipton SA et al., 1994; Quinton MS et al., 2006). Battaglia G et al. (2002) showed that selective blockade of mGlu5 metabotropic glutamate receptors is protective against methamphetamine-induced toxicity to DA terminals suggesting a role of glutamate in amphetamine toxicity while studies by Colado MI et al., (2001) showed that there is no involvement or release of glutamate and calcium with MDMA in mice.

1.6.3. Mitochondrial Dysfunction

In addition to the increased oxidative stress, more recent evidence suggests an important role of the mitochondrial electron transport chain (ETC) in mediating the toxic effects of substituted amphetamines. The first evidence of inhibition of ETC by MDMA was given by Burrows KB *et al*., (2000) where they observed significant inhibition of cytochrome oxidase activity in the substantia nigra, the nucleus accumbens, and the striatum. In line with this study Puerta E *et al*., (2010) recently reported that inhibition of complex I of the mitochondrial electron transport chain is one of

the earlier events that take place in MDMA-induced neurotoxicity in mice. Aconitase, a krebs cylcle enzyme sensitive enough to reflect in situ reactive oxygen species (ROS) generation in mitochondria was significantly decreased after MDMA treatment, supporting possible involvement of O_2^- in MDMA induced dopamine toxicity. MDMA toxicity may derive from peroxinitrite that is formed by the diffusion related reaction of O_2^- with NO. These findings are supported by the fact that mice genetically deficient in neuronal NOS and mice overexpressing human CuZn-SOD are less sensitive to MDMA toxicity compared with their wild- type counterparts. In turn lipoic acid prevents MDMA-induced 5-HT deficits in rats and dopamine deficits in mice striatum by inhibiting O_2^- production and peroxinitrite- meidiated DNA strand breakage.

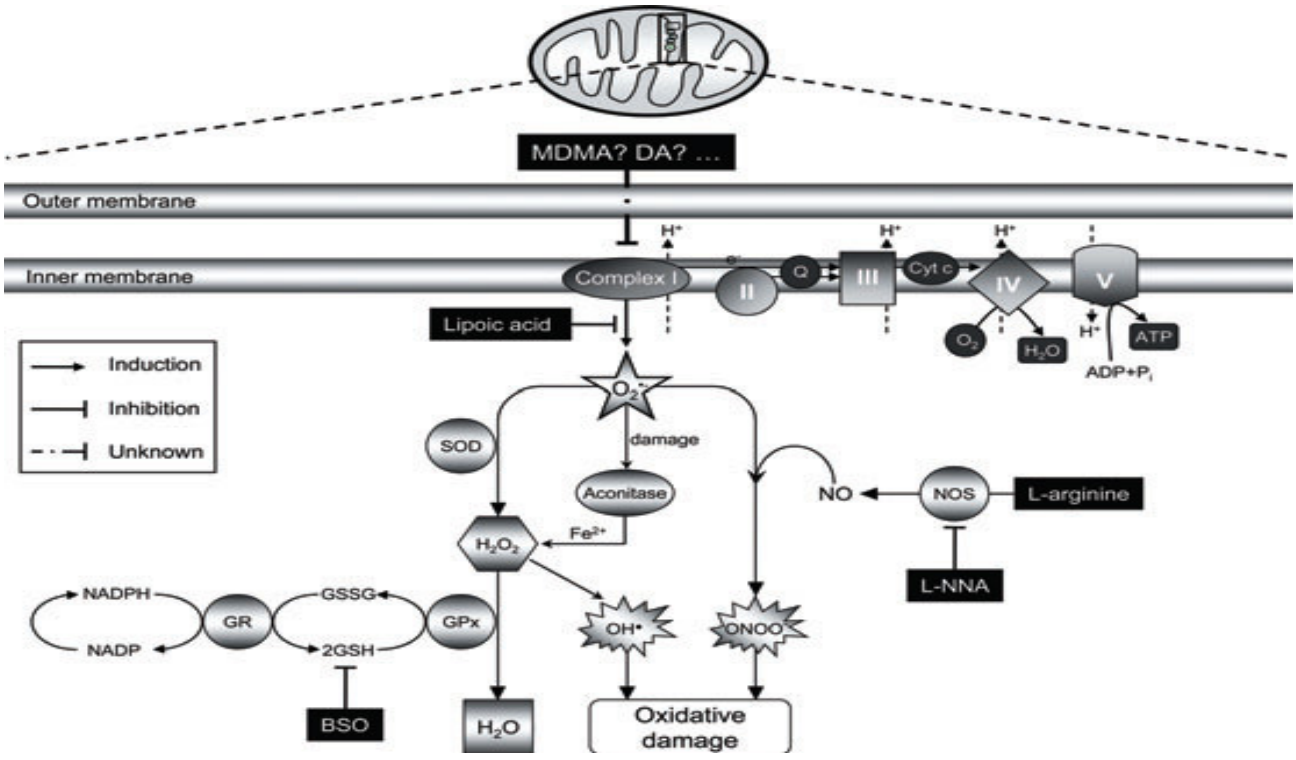

Figure 4: MDMA induced mitochondrial complex I inhibition and free radical formation (Puerta E *et al*., 2010)

1.6.4. Formation of toxic MDMA metabolites

Recent study by Yuan J *et al*., 2010 reported that dopamine is not essential for the development of methamphetamine-induced neurotoxicity giving more stress on involvement of amphetamine metabolites in amphetamine toxicity. Several studies already reported that it's not MDMA but the metabolite of MDMA that induces different neurotoxic effects. With regard to MDMA toxicity Escobedo I *et al*., (2005) showed that intrastriatal administration of MDMA at a dose much higher than the peripherally administered neurotoxic dose, does not induce neurotoxicity. So as to produce neurotoxic effects, MDMA has to get metabolised peripherally and then the metabolite of MDMA induces different neurotoxic effects. The parent compound MDMA is N-demethylated to form 3, 4 -methylenedioxyamphetamine (MDA) and O - demethylenated to form 3, 4 -dihydroxymethamphetamine (HHMA). HHMA is further O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA). In rats, N-demethylation to MDA is one of the main metabolic pathways, whereas in humans O-demethylenation to HHMA predominates. 3, 4 - Dihydroxyamphetamine (HHA) and HHMA are the precursors of neurotoxic species (De La Torre *et al*., 2004). The N- demethylation product 3,4-methylenedioxyamphetamine (MDA) which undergoes oxidation by GSH to form 5-(GSyl)-α–MeDA and might be the main neurotoxic metabolite involved in rats. Studies by Miller RT *et al*., (1996) reported that intracerebroventricular (icv) administration of 5-(glutathion-*S*-yl)-R-MeDA (720 nmol) to male Sprague-Dawley rats produced behavioral changes similar to those reported after subcutaneous adminstration of MDA and also caused short-term alterations in the dopaminergic, serotonergic, and noradrenergic systems. Pretreatment of rats with acivicin an inhibitor of γ glutamyl transpeptidase (γ-GT) increases brain uptake of 5-GSyl-R-MeDA. Thus potentiates MDA and MDMA-mediated depletions in serotonin (5-HT) and 5-hydroxylindole acidic acid (5-HIAA) concentrations in brain regions enriched in 5-HT nerve terminal axons (striatum, cortex, hippocampus, and hypothalamus) (Bai F *et al*., 2001).

The ring hydroxylation of MDMA yields products such as 6-hydroxy- MDMA (6-OH-MDMA) and 2,4,5-trihydroxymethamphetamine (THMA). The intracerebroventricular (i.c.v.) administration of 6-OH-MDMA does not produce a decrease in tryptophan hydroxylase activity (Elayan I *et al*., 1992; Zhao *et al*., 1992); however, the i.c.v. and intrastriatal administration of THMA produced 5-HT depletion and decreased tryptophan hydroxylase activity (Elayan I *et al*., 1992; Johnson M *et al*., 1992; Zhao ZY *et al*., 1992).

In mice, MDMA-induced neurotoxicity is mainly dopamine - mediated as MDMA causes the release of dopamine, which leads to the generation of reactive oxygen species as a result of dopamine oxidation. In other animal species, including humans, hepatic metabolism is a key factor involved in the production of MDMA toxicity to 5-HT-containing neurons (De La Torre *et al*., 2004).

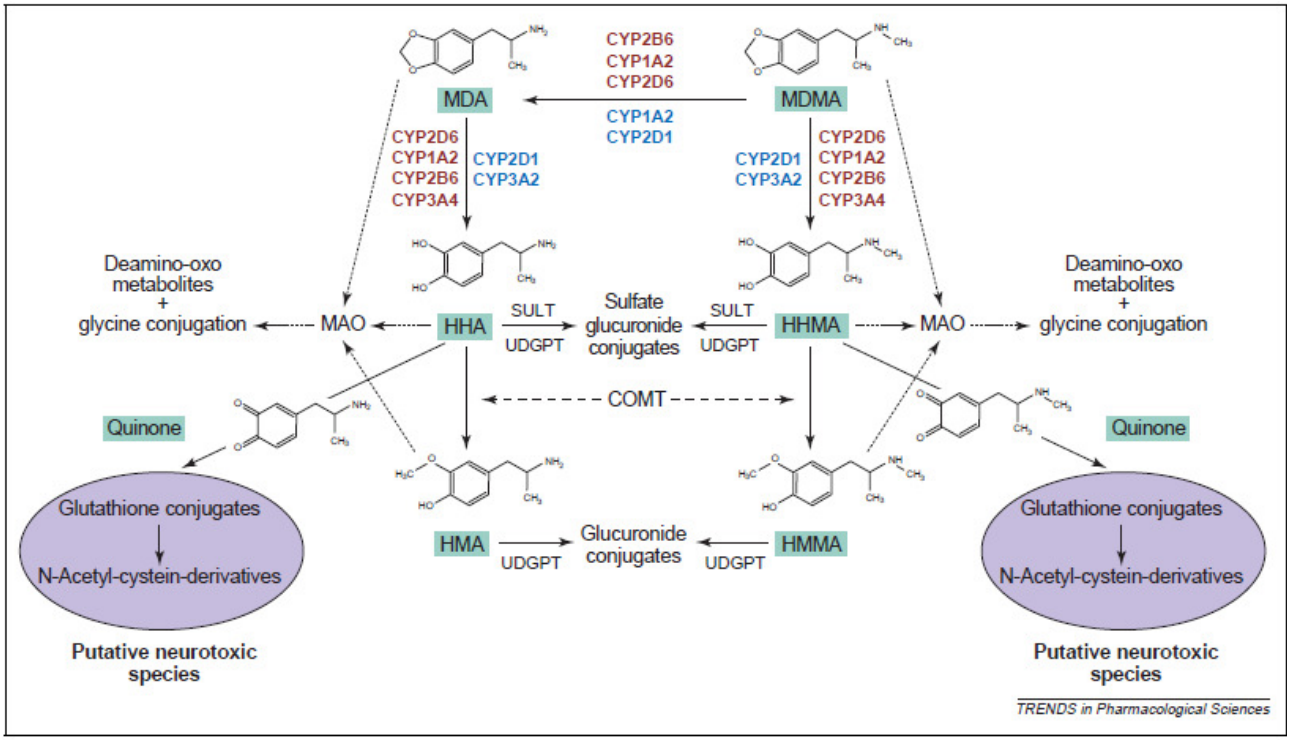

Figure 5. Pathways of 3,4-methylenedioxymethamphetamine (MDMA) metabolism in rats and in humans. Isoenzymes of cytochrome P450 (CYP) involved in the N-demethylation and O-demethylenation metabolic reactions in rats are highlighted in blue whereas those corresponding to enzymes in humans are shown in red (De La Torre R *et al*., 2004).

1.6.5. Hyperthermia

One of the mechanisms implicated in MDMA neurotoxicity is a hyperthermic response induced by the repeated administration of a drug. In rats hyperthermia results primarily from dopamine release and is influenced by dose, ambient temperature and other housing conditions. Rats housed at an ambient temperature of 11℃, MDMA produced a dose-dependent hypothermic response while at an ambient temperature of 24℃ a hyperthermic response occurred. MDMA interferes with heat loss mechanisms and consequently the higher the ambient temperature the more impaired the ability to lose heat to the environment. The metabolic rate was increased in the MDMA treated rats at an ambient temperature of both 20 and 30℃. Though MDMA causes major release of both 5-HT and dopamine, SCH23390 dopamine D1 receptor antagonist was found to be protective against MDMA induced hyperthermia. Cytokines such as interleukin 1β, interleukin-6 and tumour necrosis factor-α increase body temperature acting by direct or indirect mechanisms on the brain. After MDMA administration to rats there is an acute increase in interleukin-1β concentration in the hypothalamus and cortex (Green RA *et al*., 2004). Whereas studies by Orio L *et al*., (2004) reported that interleukin -1β could be the consequence, rather than the cause of MDMA induced hyperthermia. In mice MDMA induced hyperthermia appears to be more variable than that seen in rats. Both the dose administered and strain of mouse appears to influence the size and direction of response detected. The mechanism by which MDMA induces hyperthermia in mice appears not to involve the acute dopaminergic effects (Green RA *et al*., 2003; 2004). Maintenance of animals at low ambient temperatures (10 ℃) before and after treatment with MDMA prevents the hyperthermic response and either attenuates or eliminates the MDMA-induced neurotoxicity (Schmidt CJ *et al*., 1990; Broening HW *et al*., 1995). In contrast some agents such as fluoxetine, which provide protection against MDMA induced neurotoxicity, do not block the MDMA induced increase in temperature (Cadet JL *et al*., 2007). Taken together this evidence indicates that hyperthermia has an important modulatory role but is not an essential factor in the neurotoxicity induced by the drug.

1.6.6. Neuroinflammation

Neuroinflammation is found to be one of the factors involved in MDMA induced neurotoxicity and minocycline an anti-inflammatory drug was found to have a neuroprotective effect against MDMA induced neurotoxicity (Zhang L et al., 2006). Inflammatory response in CNS has been associated with many chronic neurodegerative conditions including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). Whether neuroinflammation is a cause or a consequence of neurologic disease remains unclear. It is observed that infection, trauma, stroke, toxins and other stimuli may cause an acute neuroinflammatory response which leads to activation of the resident immune cells (microglia) resulting in a phagocytic phenotype and the release of inflammatory mediators such as cytokines and chemokines. This acute neuroinflammatory response may trigger oxidative and nitrosative stress which is short lived and unlikely to be detrimental to long-term neuronal survival. In contrast chronic neuroinflammation includes not only longstanding activation of microglia and subsequent sustained release of inflammatory mediators, but also results in increased oxidative and nitrosative stress. Rather than serving a protective role as does acute neuroinflammation, chronic neuroinflammation is most often detrimental and damaging to nervous tissue. Thus, whether neuroinflammation has beneficial or harmful outcomes in the brain may depend critically on the duration of the inflammatory response. Neuropathological and neuroradiological studies indicate that neuroinflammatory responses may begin prior to significant loss of neuronal populations in the progression of neurodegerative diseases (Frank-Cannon TC et al., 2009).

1.7. Glial cells

Neuroglial cells of the central nervous system (CNS) include the astrocytes, oligodendrocytes, and microglia, whereas glia in the peripheral nervous system (PNS) is composed of Schwann cells. It is now well established that glial cells represent intimate partners to neurons throughout their lifespan. For example, during neurogenesis and early development, glial cells

provide a scaffold for the proper migration of neurons and growth cones, a process mediated via the synthesis and secretion of a variety of growth factors and extracellular matrix components. Glial cells also provide guidance cues for neuronal proliferation and electrical differentiation of neurons. In the adult, glial cells maintain neuronal homeostasis, synaptic plasticity, and repair (Achner M et al., 1999).

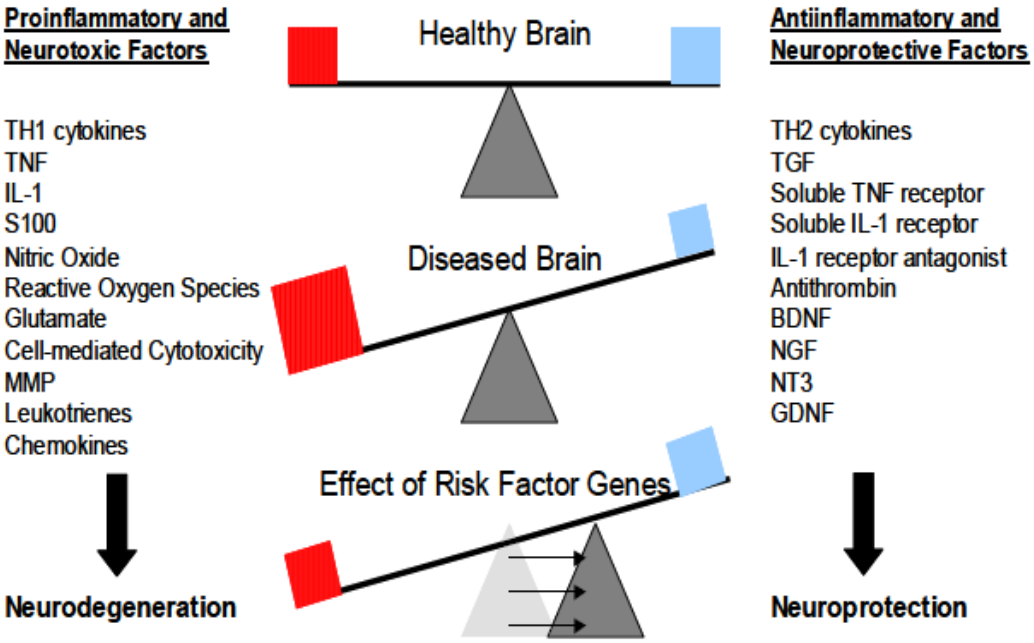

Figure 6: Balance of Inflammation. (Abbreviations: Helper T-cell Type I (TH1), Tumor necrosis factor (TNF), Interleukin-1 (IL-1), Matrix Metalloprotease (MMP), Helper T-cell Type II (TH2), Transforming Growth Factor (TGF), Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Neurotropic Factor 3 (NT3), and Glial-Derived Neurotrophic Factor (GDNF)).

In the healthy brain glia often respond to stress and insults by transiently upregulating inflammatory processes. These processes are kept in check by other endogenous anti-inflammatory and neuroprotective responses that return the brain to homeostasis. In neurodegenerative disorders, however, pro-inflammatory processes predominate and contribute to the neuronal damage observed. Several examples of proinflammatory and neurodegenerative mediators are given (left column in fig 5), as well as examples of anti-inflammatory and neuroprotective factors (right column in fig 5).

1.7.1. Microglial cells

Microglia reside in the CNS, comprise approximately 12% of the brain (depending on brain region, health, or pathology), and serve as the brain's immune defense. Analogous to the role of macrophages and lymphocytes in the periphery, one role of microglia is to act as the brain's immune defense against disease and injury. Resting ramified, microglia cell bodies are spaced throughout the CNS to avoid cell body overlap, but have been shown to be present with variable density in different brain regions. The expression of certain receptors, such as CD200 and CX3CR1 on the microglia cell surface, may interact with ligands that keep microglia in a resting state. When reacting to extracellular signals, such as the presence of pathogens, foreign material, and dead or dying cells, microglia may undergo a morphological change into an ameboid shape with short or nonexistent processes. This morphological change is also accompanied by changes in signaling and gene expression that can result in changes in surface receptor expression, the release of pro- or anti-inflammatory factors, recruitment molecules, and ROS, among others. In the developing brain and in areas of remodeling, microglia are responsible for the phagocytosis of cellular debris resulting from apoptosis and normal cell death. Microglia have been implicated as the "brain's electricians, in which the release of neurotrophic factors and anti-inflammatory cytokines from microglia has been shown to promote synaptic plasticity. In fact, the majority of microglial functions are beneficial and necessary for a healthy CNS, as activated microglia are critical for CNS wound healing. In addition, microglias have also been shown to release anti-inflammatory and trophic molecules to enhance the survival of surrounding neurons.

In contrast microglia is a predominant source of proinflammatory factors [TNF-α (tumour necrosis factor α), PGE_2 (prostaglandin E_2) and IFN-γ (interferon γ),] and oxidative stress ($^{\bullet}NO$, H_2O_2, $O^{2\bullet-}$ and $ONOO^-$/ONOOH) which are toxic to neurons. Although microglia is necessary for normal function, microglia when activated by an extensive list of pro-inflammatory stimuli, such as lipopolysaccharide (LPS), pesticides (e.g., paraquat, lindane and rotenone), disease proteins, α synnuclein, and even neuron damage, can result in disastrous neurotoxic consequences. Microglia

also has been implicated to play both causative and exacerbating roles in neurodegerative diseases. Neurodegenerative diseases are characterized by chronic and progressive neuronal loss, and pathological levels of cytotoxic substances, such as extracellular debris, elevated levels of pro-inflammatory factors, and production of reactive oxygen species, resulting in oxidative stress. These factors, in addition to the release of others that can activate and recruit microglia, support a role for microglia in diseases, such as Alzheimer's disease, PD, multiple sclerosis, amyotrophic lateral sclerosis, and HIV-associated neurocognitive disorder. Pioneering work by Mc Geer et al., (1998) discovered increased staining of the MHC class II cell surface receptor HLA-DR in the substantia nigra (SN) of postmortem PD patient brains, indicating the presence of activated microglia, and first implicating that these cells may have an active pathological role in disease. It is hypothesized that microglial activation results in selective dopamine neurotoxicity due to the inherent susceptibility of the dopamine neuron to oxidative stress. Microglia is a robust source of oxidative stress in the brain, where extracellular ROS is predominantly generated from NADPH oxidase. NADPH oxidase is a multi-subunit enzyme complex in phagocytes such as microglia, which is activated during host defence to catalyse the production of superoxide from oxygen. A variety of stimuli, including bacteria, inflammatory peptides and multiple neurotoxins activate NADPH oxidase. In addition, this enzyme complex is associated with neurodegerative disorders and neuronal damage. (Lull ME et al., 2010, Block ML et al., 2007)

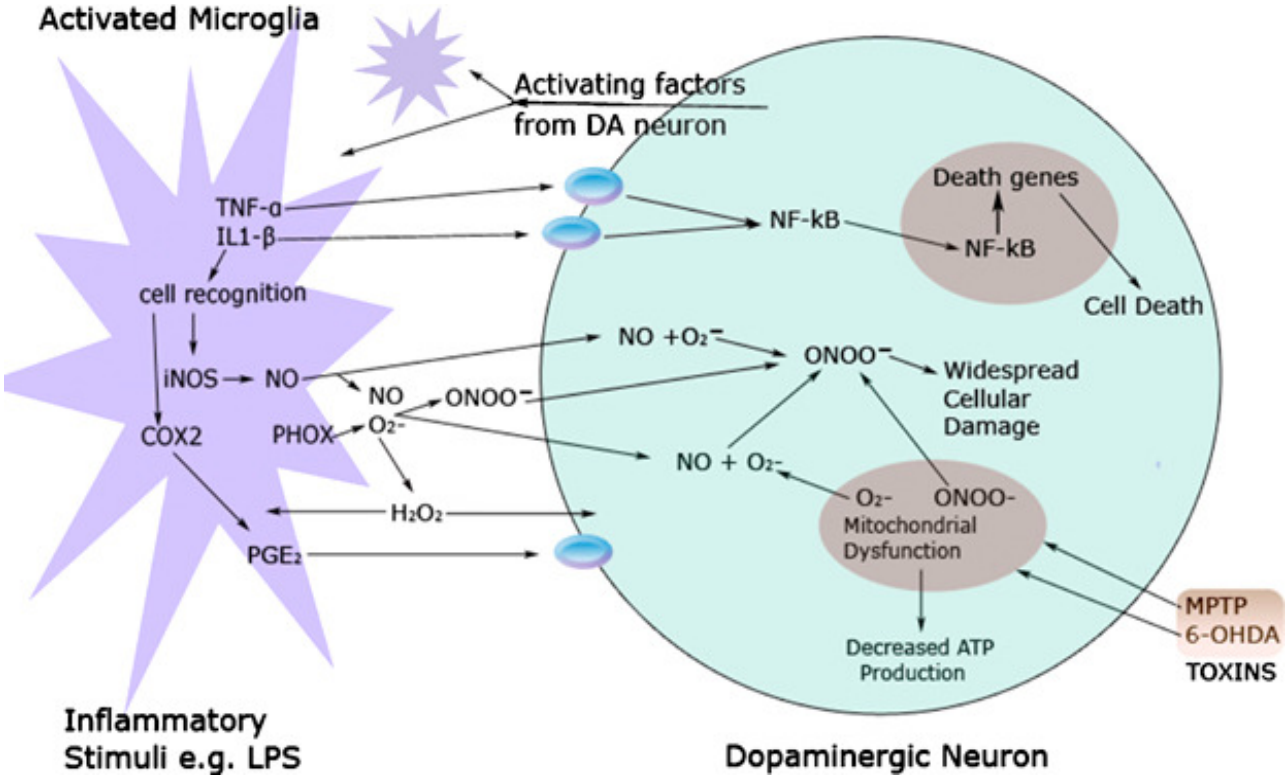

Figure 7: Release of proinflammatory factors from activated microglia and its effects on doapminergic neuron.

1.7.2. Astroglia

Astrocytes are one of the two primary types of macroglia. They comprise nearly 35% of the total CNS cell population and like microglia are found in all regions of the CNS. Histologically, astrocytes can be visualized by immunolabeling with antisera specific for glial fibrillary acidic protein (GFAP), S100b or the astrocyte specific glutamate transporters, GLT1 and GLAST (Carson MJ *et al*., 2006). In the healthy, uninjured CNS, astrocytes perform numerous functions absolutely essential for neuronal function. In case of injury, astrocytes can phagocytose injured cells after which they replace them and form a glial scar. They are slow to react to injury, but stay activated during the late recovery stage (Gehrmann J *et al*. 1995). Astrocytes produce several growth factors and regulate the induction and maintenance of neurite outgrowth, axonal guidance and synapse formation (Gee JR and Keller JN, 2005). Their most amazing function is probably their role in the tripartite synapse, which consist of the presynaptic neuron, the postsynaptic neuron and the

astrocyte itself. Astrocytes act as a physical link between the pre- and postsynaptic neurons on one side and blood capillaries on the other, which is important for maintaining the external environment for optimal functioning of the neurons. Astrocyte interactions with the cerebrovasculature endothelium play a key role in the induction and maintenance of the tight junction's characteristic of the intact BBB and can influence BBB permeability when needed (Carson MJ *et al*., 2006). Astrocytes detect synaptic activity by binding of neurotransmitters to receptors on the astrocytic membrane. By secreting vasoactive substances, astrocytes can regulate the blood flow in reaction to varying levels of neuronal activity, since an active brain region requires more oxygen and energy (Parri R and Crunelli V 2003, Benarroch EE 2005). A large body of evidence indicates that astrocytes are involved in the control of glutamate homeostasis and susceptibility of the brain to excitotoxic injury. Glutamate transporters are expressed in many different types of brain cells, but astrocytes are primarily responsible for glutamate uptake. After uptake of glutamate into astrocytes, the enzyme glutamine synthetase converts glutamate into glutamine, which is then transported into neurons where it is converted back into glutamate (Boison D et al., 2010). Besides terminating the action of glutamate, astrocytes are also responsible for the clearance of γ-aminobutyric acid (GABA) and glycine, which both are inhibitory neurotransmitters. However, maintaining synapses and uptake of neurotransmitter and thereby terminating the presynaptic signals are not the only functions of the astrocyte in the tripartite synapse. Astrocytes have G-protein coupled receptors that bind neurotransmitters, ATP or adenosine. This enables them to monitor the activity of neurons directly (Fields and Stevens-Graham 2002). Activation of these G-protein coupled receptor results in an intracellular [Ca^{2+}] rise in the astrocyte causing the release of chemical transmitters, including several neurotransmitters, ATP and D-serine (Fields RD and Stevens-Graham B 2002; Halassa MM *et al*. 2006). The secreted chemical transmitters are also called gliotransmitters and they give feedback to neurons, influencing neuronal excitability and synaptic strength. Different responses might be elicited by secreting different types of gliotransmitters, depending on the characteristics of the [Ca^{2+}] response evoked. One of these gliotransmitters is ATP, which can bind the presynaptic

neuron thereby modulating synaptic transmission. ATP also binds receptors on neighbouring astrocytes, inducing inositol triphosphate (IP_3) formation that subsequently triggers the release of Ca^{2+} stored in the endoplasmatic reticulum (ER). This way, astrocytes are capable of communicating with each other. Astrocytes can be a significant source of extracellular glutamate, which can be released by a variety of mechanisms. It has been demonstrated that Ca^{2+} elevations in astrocytes induce excitotoxic release of glutamate from these cells. It was shown that astrocyte-derived glutamate targets synaptic N-methyl-D-aspartic acid (NMDA) receptors providing a rationale explanation for the astrocyte-based control of neurotoxicity (Boison D et al., 2010).

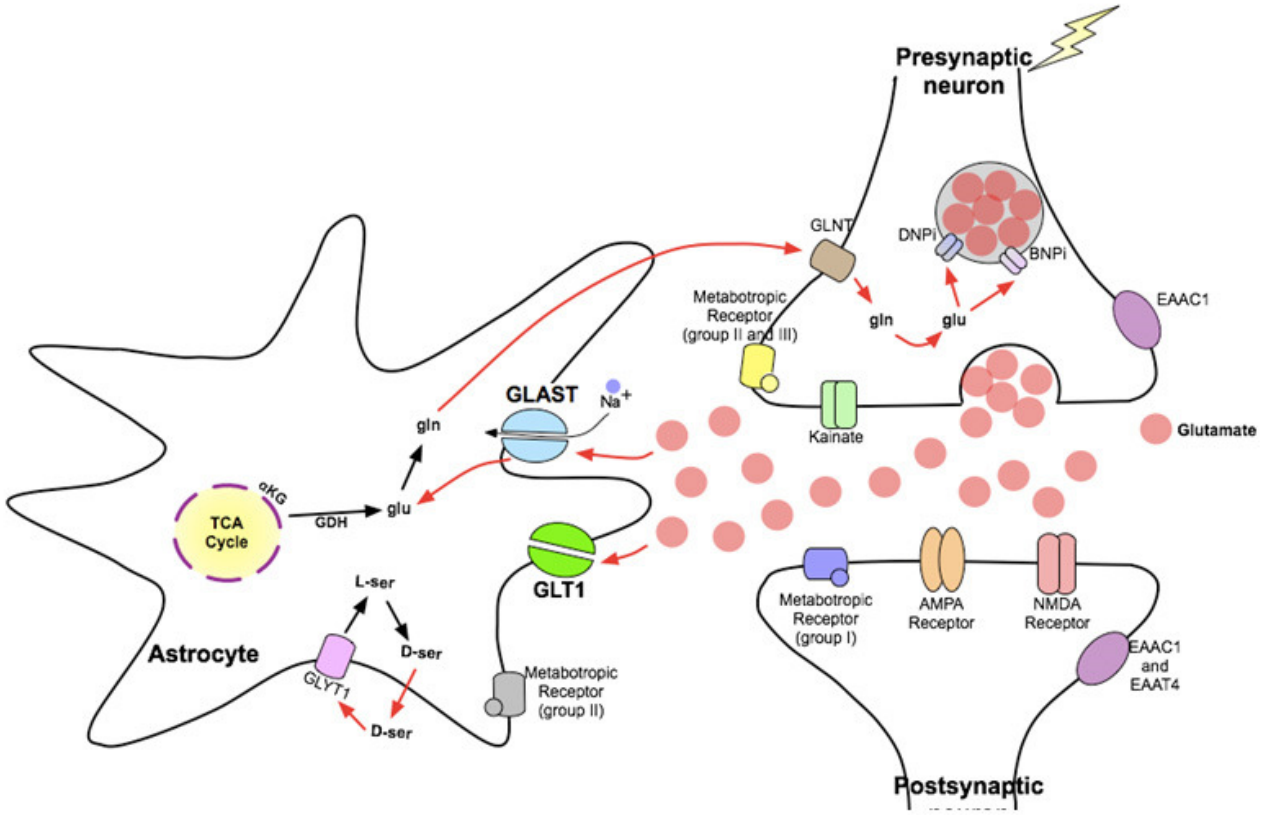

Figure 8: Glutamate transporters in astrocytes are responsible for synaptic glutamate homeostasis (Liu YP *et al.*, 2008).

1.8. Adenosine receptors and their localisation in brain

There are four types of membrane-bound adenosine receptors, named A_1, A_{2A}, A_{2B}, and A_3 receptors. All four subtypes are members of the superfamily of G-protein-coupled-receptors

(GPCRs) with seven transmembrane domains that signal through a variety of transduction mechanisms. Out of the four adenosine receptors, the A_1 receptor is the most abundant and widespread in the brain. The highest expression of A_1 receptors has been found in the cortex, cerebellum, thalamus and hippocampus. Moreover, the mRNA encoding A_1 receptor is also present in basal ganglia structures including the striatum, globus pallidus, subthalamic nucleus. It is also known that in the striatum, A_1 receptors are present on both dopaminergic nigrostriatal and glutamatergic corticostriatal terminals. Moreover, they are co-localized with dopamine D_1 receptors on GABA/dynorphin output neurons which send their terminals to the substantia nigra pars reticulata. A_1 receptors are also present in astrocytes, microglia and oligodendrocytes (Wardas J et al., 2002). A_1 receptors are most abundant in the presynaptic active zone and post-synaptic density. In contrast to the widespread distribution of A_1 receptors in the brain, A_{2A} receptors are highly concentrated in the basal ganglia predominantly located in dendritic spines and post synaptic densities of cortical and thalamic glutamatergic projections and medium spiny GABAergic neurons, where their density is about 20 times greater than elsewhere in the brain. With regard to specific neuronal populations in the striatum, A_{2A} receptors are present in striatopallidal enkephalin - expressing neurons. The same cells also express dopamine D_2 receptors; hence both A_{2A} and D_2 receptors are distributed on the same neuronal pathway. Apart from this A_{2A} receptors are also located in astrocytes and microglia cells as well as in brain blood vessel, most likely in endothelial cells. Both in-situ hybridization, binding, immunological and functional studies have concluded that A_{2A} receptors are located in limbic and neocortical regions in the brain. In conclusion A_1 receptors are mostly located presynaptically and also have a post-synaptic localization. Whereas A2A receptors are present mainly presynaptically, the striatum is exception, where A_{2A} receptors are located post-synaptically (Cunha RA et al., 2005). Due to low abundance in the brain, the role of A_{2B} and A_3 receptors has received considerably less attention. A_{2B} is expressed in intermediate levels in blood vessels, eye, median eminence, mast cells and in low levels in the adrenal and pituitary glands. At the cellular level, it has been demonstrated their biochemical existence in

neurons and glial cells (Daly JW 1977; Fredholm BB et al., 2005). A_3 is expressed at low levels in the rat or mouse brain, for example in cortex, amygdala, striatum, olfactory bulb, nucleus accumbens, hippocampus, hypothalamus, thalamus and cerebellum (Linden J *et al*., 1993; Salvatore CA *et al*., 1993; Dixon AK *et al*., 1996). At the cellular level it is expressed in neurons (Lopes LV *et al*., 2003), astrocytes (Wittendorp MC *et al*., 2004) and microglial cells (Moreau JL and Huber G, 1999; Hammarberg C *et al*., 2003).

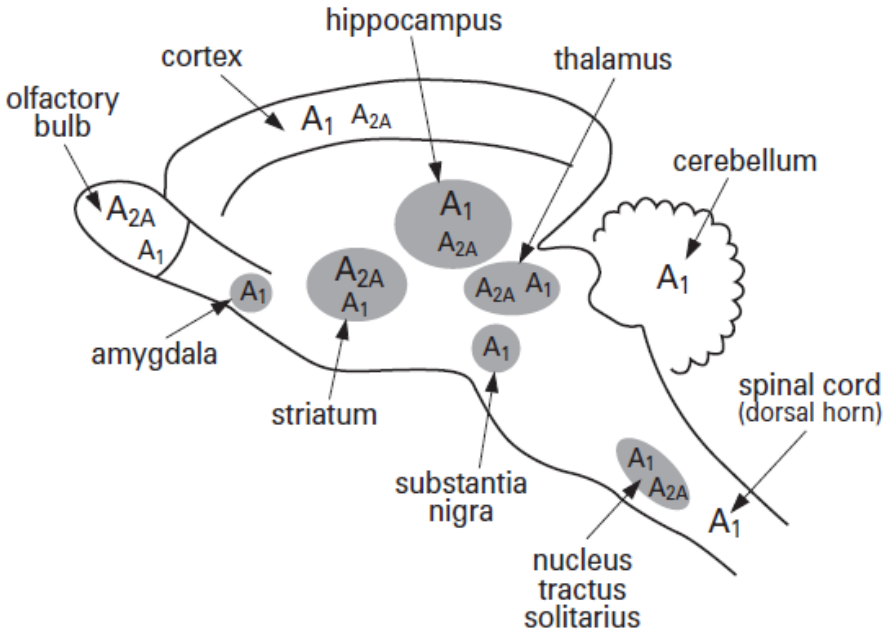

Figure 9: Distribution of adenosine A_1 and A_{2A} receptors in the brain. Bigger fonts indicate high level of expression.

1.9. Role of adenosine in the central nervous system

At a cellular level, adenosine has a neuromodulatory role on nerve activity, by modulating the release of neurotransmitters, the post-synaptic responsiveness and the action of other receptor systems (Cunha RA, 2001). The A_1 receptor inhibits synaptic transmission, acting both pre and post-synaptically in brain regions with a high concentration of these receptors, such as the hippocampus (Dunwiddie TV and Masino SA, 2001). A_1 receptor stimulation inhibits the release of most classical neurotransmitters: glutamate, acetylcholine, norepinephrine, 5-hydroxytryptmanine,

dopamine and other transmitters (Dunwiddie TV and Haas HL, 1985; Schubert P *et al*., 1986; Proctor WR and Dunwiddie TV, 1987; Barrie AP and Nicholls DG, 1993; Ambrósio AF *et al*., 1997). A_{2A} receptor mediates facilitation of the release of neurotransmitters such as glutamate (Lopes LV et al., 2002), acetylcholine (Rebola N et al., 2002) and serotonin (Okada M et al., 2001), among others. In many regions this effect is only seen if A_1 receptor is present (Lopes LV et al., 2002), but A_{2A} receptor can also facilitate the release of neurotransmitters independently of A_1 receptor, as typified by the control of the evoked release of GABA (Gubitz AK *et al*., 1996; Cunha RA and Ribeiro JA, 2000; Brooke RE *et al*., 2004). The final target of A_{2A} receptor modulation in nerve terminals seems to be P-type calcium channels (Mogul DJ *et al*., 1993; Gubitz AK *et al*., 1996). The overall neuromodulatory role of adenosine in the CNS is a balance between A_1 and A_{2A} receptor functions, because they are the two mainly expressed adenosine receptors in the brain (compared to A_{2B} and A_3) and they can be located at the same synapse (Rebola N *et al*., 2005b). In the hippocampus, the percentage of nerve terminals with A_{2A} that are simultaneously endowed with A_1 is 80% (Rebola et al., 2005a). The A_{2A} receptor has a major role in controlling A_1 receptor through intracellular transducing systems (Dixon AK *et al*., 1997; Lopes LV *et al*., 1999) or through receptor dimerization (Ciruela F *et al*., 2006). Adenosine can possess other functions such as controlling the rate of metabolism of neurons and astrocytes (Håberg A *et al*., 2000; Hammer J *et al*., 2001), axonal growth (Rivkees SA *et al*., 2001) or axonal guidance (Corset V *et al*., 2000; Stein E *et al*., 2001). Adenosine receptors can also control astrogliosis, the release of neuroactive substances (Hindley S *et al*., 1994; Ciccarelli R *et al*., 2001), inflammation (Ohta A and Sitkovsky M, 2001) and vascular resistance (Olsson RA and Pearson JD, 1990).

1.10. Adenosine and neuroprotection

Adenosine is released upon stressful situations (Fredholm BB *et al*., 2005). Consequently, a possible neuroprotective strategy is the control of the levels of adenosine by manipulation of adenosine kinase activity (Gouder N et al., 2004). This enzyme acts as key sensor and regulator of

ambient adenosine and can play a pivotal role in fine-tuning glutamatergic and dopaminergic neurotransmission, based on adenosine's activation of its receptors with opposing activities (A_1 *versus* A_{2A}) (Boison D, 2008). Gouder and collaborators reported that inhibition of adenosine kinase (thus increasing extracellular endogenous adenosine) effectively decreased chronic convulsive behavior, in an animal model of epilepsy (Gouder N et al., 2004). Increasing extracellular adenosine could be considered a suitable therapeutic target to obtain neuroprotection in other brain conditions besides epilepsy, such as ischemia (Pignataro G et al., 2007), stroke (Kowaluk EA et al., 1998; Boison D, 2006), chronic pain (McGaraughty S and Jarvis MF, 2006) or schizophrenia (Lara DR et al., 2006). However, pharmacological manipulation of adenosine kinase activity can lead to the appearance of severe side effects (Ugarkar BG et al., 2000; Gouder N et al., 2004). It has already been suggested that adenosine kinase is effective mainly under physiological conditions; whereas, in pathological states, when extracellular level of adenosine is increased, adenosine deaminase seems to play a pivotal role. It has been shown that 2-deoxycoformycin; an inhibitor of adenosine deaminase prevents histological changes in the hippocampus by decreasing the infarct area and neuronal degeneration in global forebrain ischemia in rats and gerbil (Wardas J et al., 2002). A_1 receptors can play a role in neuroprotection since their activation decreases glutamate release and hyperpolarize neurons which are found to be responsible for ischemia-induced cell death (Gerber U and Gahwiler BH, 1994; Cunha RA, 2005). Adensine A_1 receptors may provide protection by acting via pre and post-synaptic receptors. When adenosine is released it acts on presynaptic A_1 receptors and may attenuate the influx of Ca^{2+} thorugh voltage-dependent calcium channels and thus decrease the release of glutamate. By inhibiting this release, adenosine decreases the excitability of NMDA receptors and as a consequence hinders the NMDA - mediated $Ca2^{+}$ influx to neurons, the latter being the major mechanism that underlies neuroprotection. Another putative mechanism of the neuroprotective action of adenosine is related to postsynaptic adenosine A_1 receptors. By stimulating postsynaptic A_1 receptors, adenosine counteracts excessive membrane depolarization by the activation of K^{+} channels and increases in the efflux of K+, which

leads to hyperpolarization of postsynaptic neurons. As consequence, adenosine A_1 receptor stimulation diminishes the opening of voltage-dependent Ca^{2+} channels and neuronal Ca^{2+} influx. By antagonizing membrane depolarization, adenosine elevates the threshold for the opening of the NMDA receptor - operated channels, which possibly contributes to its neuroprotective action. Therefore, by stabilizing membrane potentials and maintaining intracellular Ca^{2+} homeostasis in postsynaptic neurons, adenosine may act as a neuroprotector (Wardas J et al., 2002).

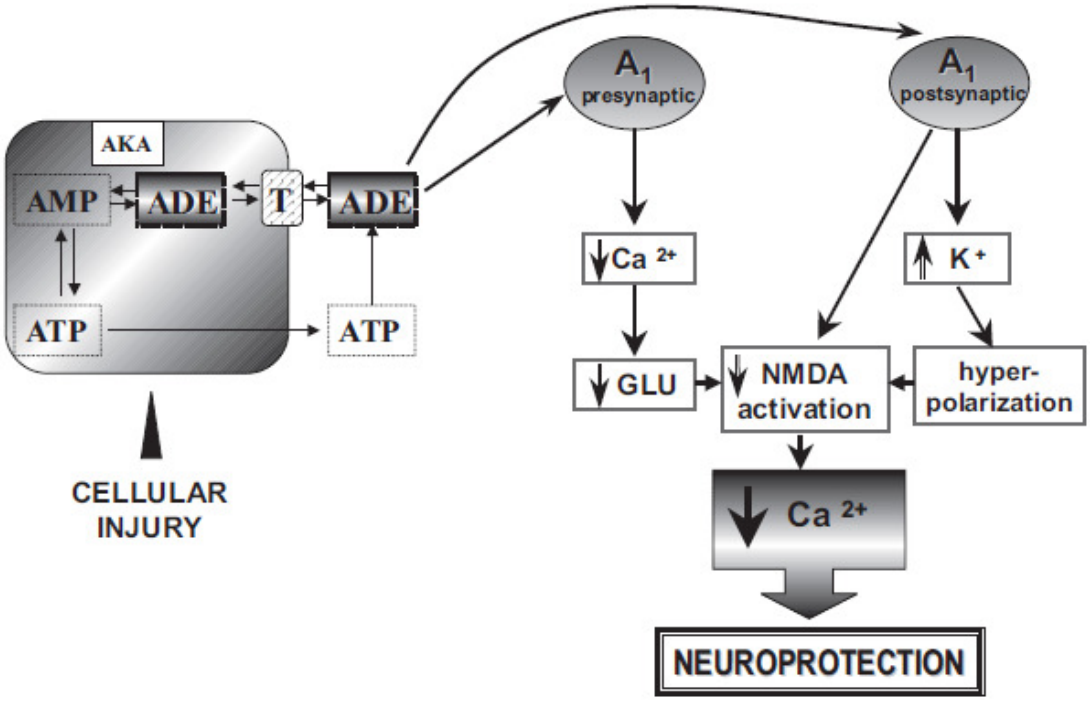

Figure 10: Schematic representation of possible mechanism responsible for the neuroprotective action of adenosine A_1 receptor agonist (AKA – adenosine kinase, ADE – adenosine deaminase) (Wardas J et al., 2002).

Thus it has been described that A_1 receptor agonists and antagonists consistently attenuate and potentiate brain damage, respectively (de Mendonça A et al., 2000). However, the use of A_1 receptor agonists as a neuroprotective strategy has several disadvantages, namely the occurrence of prominent cardiovascular effects (Olsson RA and Pearson JD, 1990; Shryock JC and Belardinelli L, 1997), the poor brain permeability of A_1 receptor agonist and their short "window of opportunity"

(Cunha RA, 2005). Given that chronic noxious stimuli cause a down-regulation of A_1 receptor and an up-regulation of A_{2A} receptor, there is a trend to emphasise the interest of A_{2A} receptor compared to A_1 receptor in neuroprotection (Cunha RA, 2005). In fact, chronic stressful stimuli cause an increased expression and density of A_{2A} receptor in animal models of Parkinson's disease (Pinna A *et al*., 2002), of epilepsy (Rebola N *et al*., 2005a), diabetes (Duarte JM *et al*., 2006) or restraint stress (Cunha GS *et al*., 2006). Most notably, A_{2A} receptor antagonists confer neuroprotection in several pathological conditions in adult animals such as upon ischemia (Monopoli A *et al*., 1998; Chen JF *et al*., 1999), or excitotoxicity (Jones A *et al*., 1998; Behan W and Stone TW, 2002). In humans there is an inverse association between caffeine consumption and Parkinson's (PD) disease (Ross GW *et al*., 2000). A_{2A} receptor antagonists are currently being developed as anti-parkinsonian drugs, since they are claimed to provide a double benefit: 1) symptomatically they prevent motor dysfunction; 2) they also provide neuroprotection (Chen JF et al., 2007). In fact, caffeine and other A_{2A} receptor antagonists provide functional protection against dopaminergic neurotoxicity and also reduce degeneration of the dopaminergic system in the MPTP model of PD (Chen JF *et al*., 2001; Xu K *et al*., 2002). Chen and colleagues reported that A_{2A} receptor - mediated control of psychomotor function and neuroprotection involves distinct cellular mechanisms, using forebrain neuronal-specific A_{2A} receptor knockout mice (Yu L *et al*., 2008). A_{2A} receptor activity in forebrain neurons is critical for control of psychomotor activity, but not for neuroprotection against brain injury, which might indicate a role of A_{2A} receptor in glial cells (Yu L *et al*., 2008). At this moment there is no consensus about the mechanisms by which A_{2A} receptor blockade confer a robust neuroprotection in noxious situations. Two leading hypotheses are currently being explored to explain the neuroprotection afforded by A_{2A} receptor blockade: control of glutamate excitotoxicity and control of neuroinflammation (Cunha RA, 2005).

1.11. Adenosine and neuroinflammation

Neuroinflammation is present in different conditions of brain damage and is a double-edged sword, possibly contributing for brain damage, but also for the repair and regeneration of brain tissue (Schwartz M and Moalem G, 2001). Adenosine is an endogenous purine nucleoside that is generated at sites that are subjected to 'stressful' conditions. Adenosine modulates a variety of glial functions, thus indirectly affecting neurons viability. Both microglia and astroglia cells are endowed with all known adenosine receptors that respond to changes in extracellular adenosine caused by traumatic and chemical insults with morphological and biochemical changes. However, the effects of adenosine on glial cells depends on various factors: the signaling pathway coupled to the specific receptor involved, the respective density of the receptor subtype (which may be subject of changes due to glial activation) and the respective affinity of the receptor for endogenous adenosine.

1.11.1. Effects of adenosine on astroglia

In response to noxious stimuli to the CNS, astrocytes undergo a process of proliferation, morphological change (hypertrophy of cell bodies, thickening and elongation of astrocytic processes) and increase the expression of glial fibrillary acidic protein. This process, which is termed astrogliosis, is associated with enhanced release of growth factors and neurotrophins that support neuronal growth but might also lead to the formation of neuronal scars (Liberto CM. *et al*., 2004). Astrocytes express all four subtypes of adenosine receptor, stimulation of which modulates various astrocyte functions. Adenosine acts at high affinity A_1 receptors to reduce astrocyte proliferation (Rathbone MP. et al., 1991). By contrast, increased occupancy of A_{2A} receptors, which is expected to occur following up regulation of this receptor secondary to hypoxia, trauma and inflammation (Cunha RA. 2005), increases astrocyte proliferation and activation (Brambilla R. et al 2003). This indicates that adenosine might be a key factor in inducing astrogliosis following ischemic events. Adenosine at high concentrations induces astrocyte cell death by apoptosis in

vitro, via mechanisms involving both the activation of the A_3 receptor and adenosine internalization and metabolism (Dare E et al., 2007).

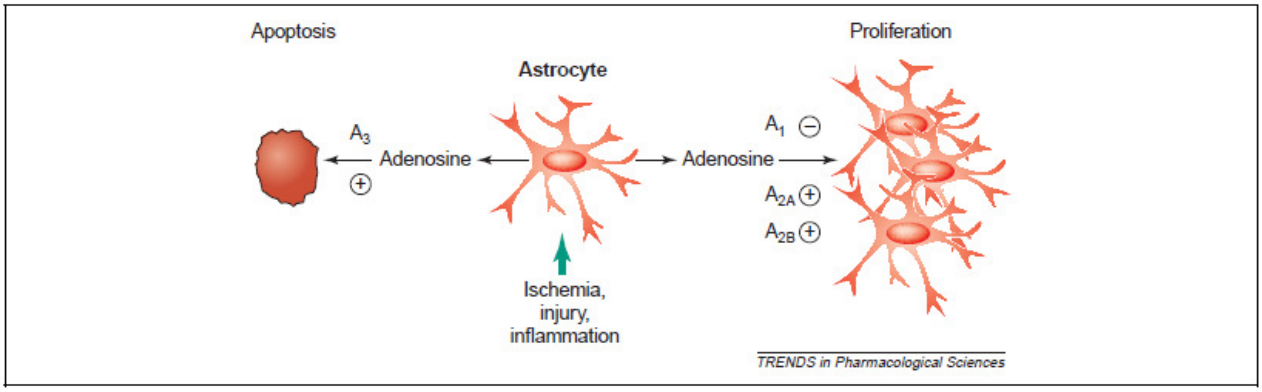

Figure 11: Regulation of astrocyte proliferation and apoptosis by adenosine receptors. (Hasko G et el., 2007)

In addition to regulating the proliferation and survival of astrocytes, adenosine has potent effects on the secretory functions of these cells. Stimulation of A_1 receptors causes the release of nerve growth factor (NGF) (Ciccarelli R. *et al*., 1999) and, thus, appears to have an important role in supporting neuronal survival and growth. A_{2A} receptor stimulation inhibits the expression of inducible nitric oxide synthase (iNOS), and thus the production of nitric oxide (NO), by astrocytes. The production of NO by iNOS in the brain seems to contribute to the pathophysiology of many CNS diseases (Licinio J. *et al*., 1999), so the inhibition of NO formation by adenosine might be an important protective mechanism during inflammatory conditions in the brain. Finally, A_3 receptor stimulation induces the synthesis of a neuroprotective chemokine called chemokine (C-C motif) ligand 2 (CCL2; known formerly as monocyte chemoattractant protein 1) by astrocytes (Wittendorp, MC. *et al*., 2004). Taken collectively, adenosine appears to alter astrocyte function in ways that are consistent with a neuroprotective role. Nevertheless, adenosine might also aggravate tissue injury by inducing excessive astrogliosis. It should be remembered that astrocytes are an important component of the blood brain barrier (BBB) and are directly involved in the control of the BBB permeability via release of mediators. Recent experiments performed with an experimental

BBB in vitro model have not supported the hypothesis of a major role of adenosine receptor subtypes in the regulation of BBB permeability (Dare E *et al*., 2007).

1.11.2. Effects of adenosine on microglia

Microglia the immunocompetent cells of the central nervous system, react to chemical and structural changes in their environment with morphological and biochemical changes, switching from a silent ramified state to an active phagocytosing macrophage like phenotype (Stoll G. et al., 1999). Most neurological disorders involve activation and, possibly, dysregulation of microglia. Microglia expresses A_1 receptors, A_{2A} receptors and A_3 receptors, but there is no evidence that they contain A_{2B} receptors. Adenosine stimulates the proliferation of naive microglial cells through a mechanism that involves the simultaneous stimulation of A_1 receptors and A_2 receptors. Although the proliferation and/or apoptosis of microglia are regulated by several adenosine receptors, the secretion activity of these cells appears to be stimulated by A_{2A} receptors. For example, A_{2A} receptor stimulation up regulates cyclooxygenase 2 (COX-2) and the release of prostaglandin E2 (PGE2), which might indicate a proinflammatory role of A_{2A} receptor stimulation (Feibich BL. *et al*., 1996). Furthermore, A_{2A} receptor activation induces the synthesis and release of NGF (Heese, K. *et al*., 1997). Interestingly recent finding by Orr AG et al., (2010) showed that A_{2A} receptors are expressed on microglia specifically when they are activated. They reported that A_{2A} receptor stimulation causes decrease in ramification of microglia and thus activation of microglia. Also A_{2A} receptor stimulation retracts microglia from injury site. Although microglia contains A_3 receptors, the stimulation of which results in increased phosphorylation of extracellular signal-regulated kinase 1, 2 (ERK1, 2) (Hammarberg C. et al., 2003), the role of A_3 receptor stimulation in regulating microglial function is unclear. In summary, adenosine appears to have both pro-inflammatory and anti-inflammatory effects, and it is difficult to provide a clear picture of how adenosine affects microglial functions.

2. Aims of the study

2.1. Effect of caffeine on MDMA induced neurotoxicity

3, 4 methylendioxymethamphetamine (MDMA, ecstasy) is a popular recreational drug. Ecstasy tablets are often taken with caffeinated beverages or contaminated with caffeine in varying amounts. Previous studies showed that caffeine enhances hyperthermia and tachycardic response induced by MDMA in rats (Mc Namara N *et al*., 2006, 2007). Also studies by Camarasa et al., (2006) found potentiation of MDMA induced decrease in number of serotonergic transporter level by caffeine. Microglial and astroglial activation plays an important role in neurotoxicity and represent an early step in MDMA-induced neurotoxicity (Thomas et al., 2004; Zhang et al., 2006). During my PhD training i have studied whether association of caffeine at dose equivalent to those consumed by human with MDMA affects MDMA - induced hyperthermia, neuroinflammatory and neurotoxic effects toward dopaminergic neurons in mice.

To pursue this objective, the following experiments were carried out:

● Assessment of inflammatory response in SNc and striatum studied through analysis of GFAP and CD11b immunohistochemistry as markers of astroglia and microglia.

● Assessment of dopaminergic neuronal damage in SNc studied by tyrosine hydroxylase (TH) immunohistochemistry.

● Modification in MDMA induced hyperthermia assessed by measuring rectal temperature.

2.2. Effect of A_1/A_{2A} receptor antagonists on MDMA induced neurotoxicity

In the first part of the study we showed that caffeine a non-specific adenosine A_1 and A_{2A} receptor antagonist enhances MDMA induced microglial and astroglial activation in mice striatum (Khairnar et al., 2010). In order to evaluate involvement of A_1 and A_{2A} receptors in caffeine modulation of MDMA-effects we searched for the effect of selective adenosine A_1 and A_{2A} receptor

antagonists on MDMA induced gliosis in SNc and striatum. Moreover we evaluated dopaminergic neuronal damage after MDMA in the substantia nigra pars compacta (SNc) and the influence of A1 and A2A receptor antagonists on this event. We also tested the effect of these adenosine receptor antagonists on MDMA induced hyperthermia since several studies have correlated this effect with MDMA induced neurotoxicity (Camarasa et al., 2006; Mc Namara *et al*., 2006; Vanattou-saifoudine N et al., 2010).

To pursue this objective, the following experiments were carried out:

● Assessment of inflammatory response in SNc and striatum studied through analysis of GFAP and CD11b immunohistochemistry as markers of astroglia and microglia.

● Assessment of dopaminergic neuronal damage in SNc studied by tyrosine hydroxylase (TH) immunohistochemistry.

● Modification in MDMA induced hyperthermia assessed by measuring rectal temperature.

The results of the present study provide important insights to mechanism involved in increasing MDMA induced glial activation by caffeine.

3. Material and Methods

3.1. Drugs

MDMA–HCl (synthesized by Prof. Plumitallo Department of Pharmaceutical Chemistry and Technology, University of Cagliari) was dissolved in saline. Caffeine (Sigma–Aldrich, Milan, Italy) was dissolved in water, DPCPX (Sigma–Aldrich, Milan, Italy) was suspended in 0.5% Tween 80; SCH 58261 (kindly provided by Prof. Baraldi, Ferrara) was suspended in 0.5% Methyl cellulose.

3.2. Animals

Adult male C57BL/6J mice, 3 months old (20–25 g; Charles River, Milan, Italy) were maintained at a constant temperature (21 ± 1°C) in 12-h light/dark cycles (lights on at 08:00), and given food and water ad libitum. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the ethics committee of the University of Cagliari.

3.3. Treatment

Mice received repeated administrations of vehicle or MDMA (4 x 20 mg/kg, intraperitoneally, (i.p)) at 2 h intervals alone or in combination with Caffeine (10mg/kg, i.p.), SCH 58261 (0.5 mg/kg, i.p.) or DPCPX (0.5 mg/kg, i.p.) 30 min before the first and third administration of MDMA. On second day, mice received two administrations of Caffeine, SCH 58261, DPCPX or saline at 12 h interval and on third day one further administration. Mice were sacrificed 48 h after last administration of MDMA for immunohistochemical studies.

3.4. Immunohistochemistry

Animals were anaesthetized with chloral hydrate (450mg/kg, i.p.) and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4). Brains were isolated and kept in fixing

solution for 2h and later in PBS plus sodium azide. Coronal sections (50 µm thick) of mice brain were cut on a vibratome and immunostained for tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP) and CD11b in order to analyze dopaminergic neuronal damage, astroglial and microglial activation respectively. Immunohistochemistry was carried out in free-floating sections with standard avidin–biotin (ABC; Vector Laboratories, UK) immunohistochemical protocols. Sections were treated with hydrogen peroxide to block endogenous peroxidases and then incubated overnight with the following specific primary antisera: polyclonal rabbit anti-TH (1:1000, Biomol), monoclonal mouse anti-GFAP (1:400; Sigma–Aldrich) and monoclonal rat anti-CD11b (1:1000; Serotec, UK). For visualization it was used 3, 3 - diaminobenzidine as chromogen. After getting colour these sections were mounted on chromealum-gelatine coated slides dried and dehydrated with 70%, 96% and 100% ethanol.

3.5. Image Analysis

Images were captured under constant light conditions using a PixeLink PL-A686 camera at 10X magnification for the SNc and at 20X magnification for the striatum. For each animal, three sections from the SNc (A = -2.92, -3.28, -3.64 mm from bregma, according to Mouse brain Atlas by Paxinos and Franklin 2001), and three sections from striatum (A = 1.10, 0.74, 0.38 mm from bregma), were analyzed for each protein evaluated in the study.

GFAP immunoreactivity in the SNc was quantified by counting the number of positive cells in the entire left and right part of this structure. For quantification of GFAP immunoreactivity in the striatum, one dorsolateral and one ventromedial portion were analyzed from both the left and right parts using PixeLink image analysis software. Analysis of CD11b immunoreactivity in the SNc and striatum was done with the analysis software SCION Image (Scion Corporation, Frederick, MD, USA). Within each frame, the area occupied by gray values above the threshold was automatically calculated.

In order to obtain averages for GFAP and CD11b, values were normalized with respect to vehicle. All values were expressed as mean ± SEM and were statistically analyzed with a one-way ANOVA followed by the Newman–Keuls post hoc test.

3.6. Stereological quantification of TH-immunoreactive neurons

TH-immunoreactive (TH-ir) neurons in the substantia nigra pars compacta (SNc) were quantified in both hemispheres of each brain using the stereological principles as described previously (Ossowska et al., 2005). Systemic uniform random sampling was used to choose the sections. The first sampling item was taken at random from the frontal part of the substantia nigra (ca. -2.80 mm from the bregma according to Paxinos and Franklin, 2001) and all the remaining sampling items were taken at a fixed distance from the previous one. At least 5–6 sections through the entire length of the substantia nigra were sampled. All stereological counting procedures were performed using a microscope (Leica, DMLB; Leica, Denmark) equipped with a projecting camera (Basler Vision Technologies, Germany) and a microscope stage connected to an *xyz* stepper (PRIOR ProScan) controlled by a computer using the new CAST Visiopharm (Denmark) software. The SNc region was carefully outlined under lower magnification (5x) and its volume was estimated using Cavalieri's principle (Gundersen and Jensen, 1987). The total number of TH-ir neurons was unbiasedly estimated under higher magnification (63x) using randomized meander sampling and optical dissector method. TH-ir neurons were counted only when present completely or partially inside the frame and when they did not touch any of the red exclusion lines. Neurons which touched green inclusion lines were counted. The cut thickness of sections was 50 µm and the optical dissector height was 12 µm. The top (13 µm) and bottom (25 µm) layers that shrunk during staining procedure were discarded. In each sampled area the dissector position was adjusted. The sampling area covered 20% of the region of interest. The counting frame (8302, 8 μm^2) applied the exclusion and inclusion lines and unbiased counting was performed by an experimenter blinded to the treatment. The results are presented as the mean ±SEM from 4-6 mice per each group.

4. Results

4.1. Inflammatory response in SNc and striatum

4.1.1. GFAP Immunoreactivity in the Striatum and SNc

GFAP immunoreactivity was low in both the striatum and SNc in the basal condition (Fig. 8.1, 8.2, 8.6 and 8.7). It was significantly higher in the striatum after treatment with MDMA (20 mg/kg, i.p.), MDMA plus caffeine (10mg/kg, i.p.), MDMA plus SCH58261 (0.5mg/kg, i.p.) and MDMA plus DPCPX (0.5 mg/kg, i.p.) compared with vehicle ($P < 0.001$) (Fig 8.1, 8.6A and 8.6B). Post hoc analysis indicated that administration of DPCPX and Caffeine in MDMA-treated mice was associated with significantly higher GFAP immunoreactivity in the striatum (Fig. 1 and 6B) compared with mice treated only with MDMA ($P < 0.001$) whereas administration of SCH58261 in MDMA treated mice did not altered GFAP immunoreactivity in striatum (Fig 6A). Treatment with MDMA, MDMA plus Caffeine, MDMA plus DPCPX and MDMA plus SCH 58261 did not alter GFAP immunoreactivity in the SNc compared with vehicle (Fig. 8.2 and 8.7). Caffeine, SCH 58261 and DPCPX alone did not modify GFAP both in striatum and SNc (data not shown).

4.1.2. CD11b Immunoreactivity in the Striatum and SNc

Similar, to GFAP, CD11b immunoreactivity was low in both the striatum and SNc in the basal condition (Fig. 8.3, 8.4, 8.8 and 8.9). It was significantly higher in both striatum (Fig. 8.3, 8.8A and 8.8B) and SNc (Fig. 8.4 and 8.9) after treatment with MDMA (20 mg/kg, i.p.), MDMA plus caffeine (10 mg/kg, i.p.), MDMA plus SCH58261 (0.5 mg/kg, i.p.) and MDMA plus DPCPX (0.5 mg/kg, i.p.) compared with vehicle ($P < 0.05$). Post hoc analysis indicated that administration of SCH58261 in MDMA-treated mice was associated with significantly higher CD11b immunoreactivity in the striatum and in the SNc as compared to mice treated only with MDMA ($P < 0.05$) (Fig 8A and 9A) in contrast caffeine and DPCPX potentiated CD11b immunoreactivity only

in striatum ($P < 0.05$) but not in SNc (Fig. 8.3,8.8B and 8.9B). Caffeine, SCH58261 and DPCPX alone did not modify CD11b levels both in striatum and SNc (data not shown).

4.2. Neuronal damage in SNc

4.2.1. TH Immunoreactivity in SNc

Repeated administration of MDMA (20 mg/kg, i.p.), MDMA plus caffeine (10mg/kg, i.p.), MDMA plus SCH58261 (0.5mg/kg, i.p.) and MDMA plus DPCPX (0.5mg/kg, i.p.) significantly decreased number of TH immunopositive cells compared with vehicle in SNc ($P < 0.05$) (Fig. 8.5 & 8.10). Post hoc analysis indicated that treatment with caffeine (10mg/kg, i.p.), SCH58261 (0.5 mg/kg, i.p.), and DPCPX (0.5 mg/kg, i.p.) in MDMA treated mice did not further decreased number of TH immunopositive cells as compared to MDMA treated mice.

4.3. MDMA induced hyperthermia

Repeated administration of MDMA (20 mg/kg, i.p.), MDMA plus caffeine (10mg/kg, i.p.), MDMA plus SCH58261 (0.5mg/kg, i.p.) and MDMA plus DPCPX (0.5mg/kg, i.p.) induced significant increase in rectal temperature as compare to vehicle treated mice ($P < 0.05$) (Fig. 8.11 & 8.12). Post hoc analysis indicated that treatment with caffeine (10mg/kg, i.p.), SCH58261 (0.5 mg/kg, i.p.), and DPCPX (0.5 mg/kg, i.p.) in MDMA treated mice did not further increased rectal temperature as compare to MDMA treated mice.

5. Discussion

The results of the present study showed that acute repeated administration of MDMA induced a neuroinflammatory process in mice, characterized by microgliosis in the striatum and SNc and astrogliosis in the striatum of mice. Caffeine, when given together with MDMA, potentiated the activation of microglia and astroglia in the striatum. Caffeine is a safe substance present in beverages such as coffee, tea, soft drinks, and energy drinks. However, as shown by the present study, caffeine taken with MDMA at doses similar to those that may be taken for recreational use with energy drinks may exacerbate MDMA neuroinflammation posing serious health consequences for consumers of this drug of abuse.

As caffeine is non-specific A_1/A_{2A} receptor antagonist we also tested influence of specific A_1 and A_{2A} receptor antagonists on MDMA induced gliosis in a separate set of experiment. We found that treatment with the A_{2A} receptor antagonist SCH58261 potentiated MDMA - induced microgliosis in both the striatum and SNc, in contrast, the A_1 receptor antagonist DPCPX potentiated both microgliosis and astrogliosis in striatum but not in SNc. Thus the results of the present study indicate a role of adenosine A_1 receptor in the modulation of both astroglia and microglia induced by MDMA, whereas A_{2A} receptors modulate MDMA-induced microglia only, indicating that antagonism of A_1 receptors better correlates to the effect of caffeine. Moreover, while both adenosine receptors are involved in neuroinflammatory effects neither treatment with caffeine nor A_1 and A_{2A} receptor antagonism interfere with MDMA-induced dopamine neuron degeneration and hyperthermic effect.

Adenosine is an important neuromodulator whose concentration increases in the presence of acute and chronic brain insults with a preferential activation of A_1 or A_{2A} receptors at low or high adenosine levels respectively (Cunha RA. 2005). Glial cells are endowed with adenosine receptors and depending on the type of insult, whether it is acute or chronic, specific adenosine A_1 or A_{2A} receptors are stimulated producing either proinflammatory or anti-inflammatory effect (Cunha RA.

2005). In the presence of an acute insult, the adenosine A_1 receptor, which is stimulated at lower levels of adenosine, reduces production of inflammatory cytokines and the increase of free radicals by inhibiting astrocyte proliferation (Cunha RA. 2005, Hasko *et al*., 2005). Moreover, stimulation of the A_1 receptor enhances production of important neuroprotective substances, such as NGF, TGF-β1 and S100 β, in cultured astrocytes (Dare E *et al*., 2007). In addition, microglial cell activation was enhanced in A_1 receptor null mice and A_1 receptor deficiency increased proinflammatory responses (Tsutsui S *et al*., 2004). Thus we may hypothesize that caffeine and DPCPX after acute administration of MDMA by antagonizing the effect of adenosine A_1 receptors, may potentiate MDMA induced astrogliosis and microgliosis in mice striatum.

Similarly to the A_1, the A_{2A} receptors on glial cells exerts complex actions on neuroinflammation, however, differently from A_1 receptors, A_{2A} receptors have been found in microglia cells whereas their presence in astroglia cells and their control in astrogliosis is controversial (Melani A *et al*., 2009; Brambilla R *et al*.,2003; Fiebich BL *et al*., 1996; Alloisio S 2004). Recent findings by Orr AG et al., (2010) showed that A_{2A} receptor stimulation retracts microglia from injury site and inhibits activation of microglia, thus antagonists may increase it which is in line with the present findings where A_{2A} receptor antagonist potentiated MDMA induced microglial activation. Our results by showing that A_{2A} receptor antagonism affects microglia but not astroglia activation evidence the inability of A_{2A} receptors to control astrogliosis and suggest that the A_{2A} receptor needs to act in concert with the A_1 receptor to trigger astrogliosis. Interestingly, previous results by Brambilla R *et al*., (2003) have shown the inability of A_{2A} receptor alone to control astrogliosis in cell culture.

Previous studies by Granado N *et al*., 2008 reported that MDMA given in similar doses and regimen produced dopaminergic neuronal damage in the SNc of mice. Similarly, the current study shows a decrease in the number of TH- immunoreactive neurons in SNc after MDMA, however, neither treatment with caffeine nor with SCH58261 and DPCPX further decreased these TH-ir neurons in SNc.

An important interplay in neuron/glia communication and in neurotoxicity is played by glutamate whose release is controlled by adenosine A_1 and A_{2A} receptors in an opposite way. In addition several studies (Blum D *et al*., 2003; Li Y *et al*., 2006; Dai SS *et al*., 2010) demonstrate a dual role of A_{2A} receptors on neuroinflammation depending on the levels of glutamate. At low glutamate concentration, stimulation of A_{2A} receptors attenuated brain damage by inhibiting inflammatory cytokines expression and iNOS activity. In contrast A_{2A} receptor activation worsens inflammatory effects at high glutamate concentration (Dai SS *et al*., 2010). However, glutamate release is not supposed to be involved in MDMA neurotoxicity in mice (Colado MI *et al*., 2001, Quinton MS *et al*., 2006) and therefore actions on receptors controlling its release do not appear to influence MDMA induced neurotoxicity.

The results of the present study show that although A_1 and A_{2A} receptor blockade increase glial activation induced by MDMA, it does not actually worsen dopamine neuron degeneration induced by MDMA suggesting that adenosine receptor blockade might increase neuron vulnerability without favoring overt neurotoxicity.

MDMA induces consistent hyperthermia in rats though much more variable responses have been seen in mice (Green AR *et al*., 2003). Moreover, it has been reported that pretreatment with caffeine in MDMA-treated rats increases hyperthermia (McNamara R *et al*., 2006) and SCH58261 but not DPCPX is involved in potentiating MDMA induced hyperthermia by caffeine in the same species (Vanattou-Saïfoudine N *et al*., 2010).

An increase in body temperature has been suggested to be a contributing factor to MDMA-induced toxicity (Mc Namara R *et al*., 2006), it is therefore of particular interest that in the present study while a significant increase in temperature with MDMA was observed, neither caffeine nor SCH58261 and DPCPX further increased MDMA induced hyperthermia and dopamine neuron degeneration although they increased gliosis. This result may therefore suggest that while increase in body temperature may be necessary for potentiation of neurotoxic effects by MDMA, increase in neuroinflammatory effects appears to not be correlated with changes in this parameter.

The reason of the discrepancy between our results and those on adenosine A_{2A} receptor involvement in hyperthermia induced by caffeine in the presence of MDMA (Vanattou-Saïfoudine N *et al*., 2010), might be due to the different species used in the two studies (rats versus mice) which have different susceptibility to temperature changes by MDMA (Green AR *et al*., 2003). On the other hand in the same report Vanattou-Saïfoudine N *et al*., (2010) reported that caffeine provokes a dopamine D_1-receptor dependent exacerbation of MDMA-induced hyperthermia, suggesting that caffeine promotes post-synaptic D_1 receptor-dependent responses and that MDMA-induced neurotoxicity and hyperthermia are associated to dopamine D_1 responses.

6. Conclusions

Co-administration of MDMA and caffeine has been suggested to interact at the level of metabolism (through CYP1A2) or processes like absorption, distribution or elimination, although an interaction between the two substances at the level of catecholamine and serotonin has been envisaged as the main mechanism of interaction (Vanattou-Saifoudine N *et al*., 2010). The results of our study provide an important knowledge on the role of the specific adenosine receptor subtypes involved in modulatory effect of caffeine on MDMA-induced neuroinflammatory responses. Thus evidencing that the interaction existing between MDMA and caffeine in the mediation of glial activation but not dopamine neuron degeneration or hyperthermia in mice are due to an action on specific adenosine A_1 and A_{2A} receptors, although antagonism of A_1 receptor better correlates to potentiation of MDMA induced glial activation by caffeine.

These findings may be important to address clinical investigations of potential severe side effects associated with the combined use of caffeine and MDMA in humans.

7. References

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8. Figures

Effect of caffeine on MDMA induced GFAP (Astroglia) immunoreactivity in striatum

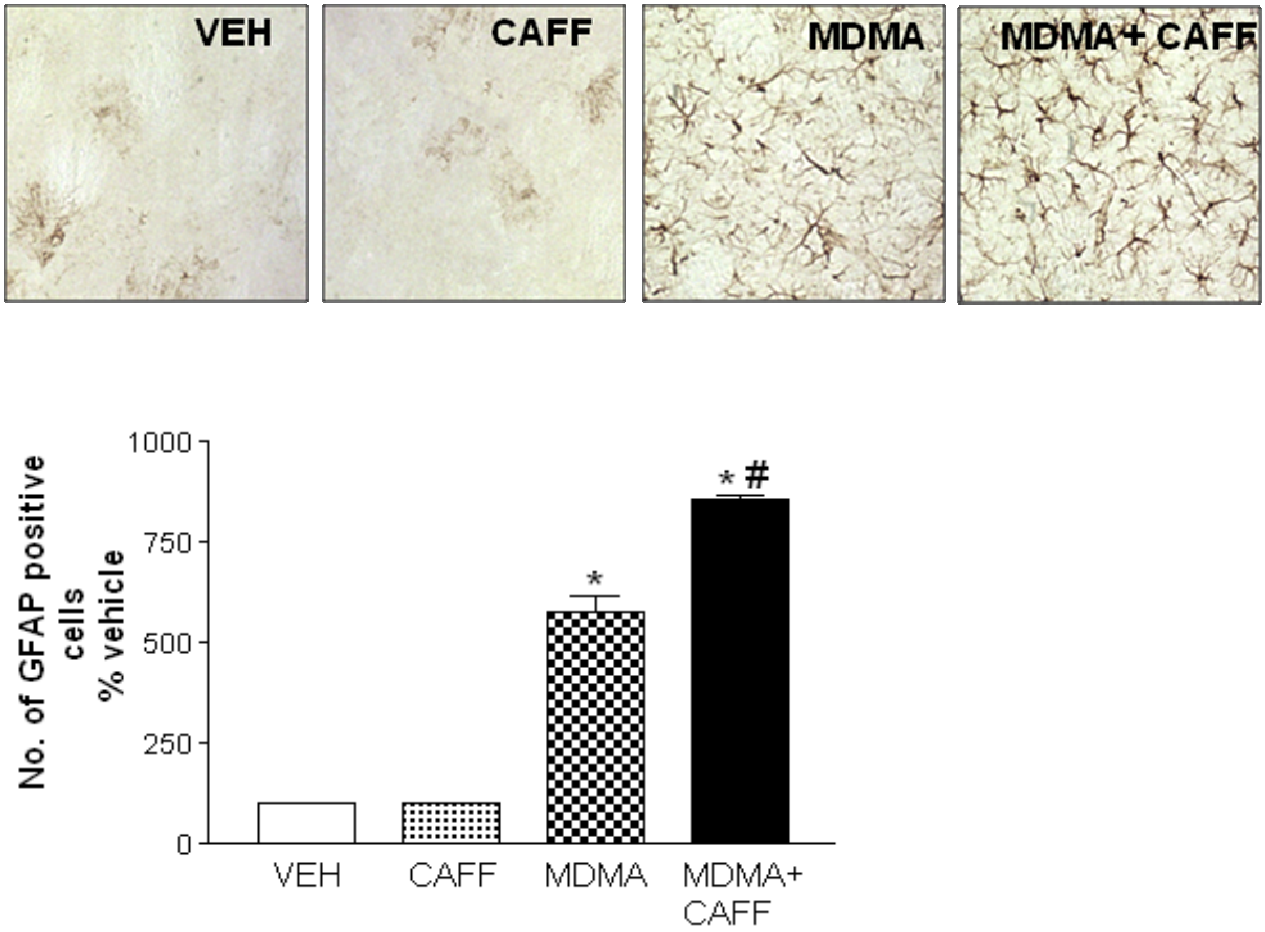


Figure 8.1: Effect of repeated administration of MDMA (20mg/kg, i.p.) and caffeine (10mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker. Representative images and histogram from the striatum immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM. *P < 0.001 versus vehicle treated group; $^{\#}P < 0.001$ versus the group treated with MDMA alone.

Effect of caffeine on MDMA induced GFAP (Astroglia) immunoreactivity in SNc

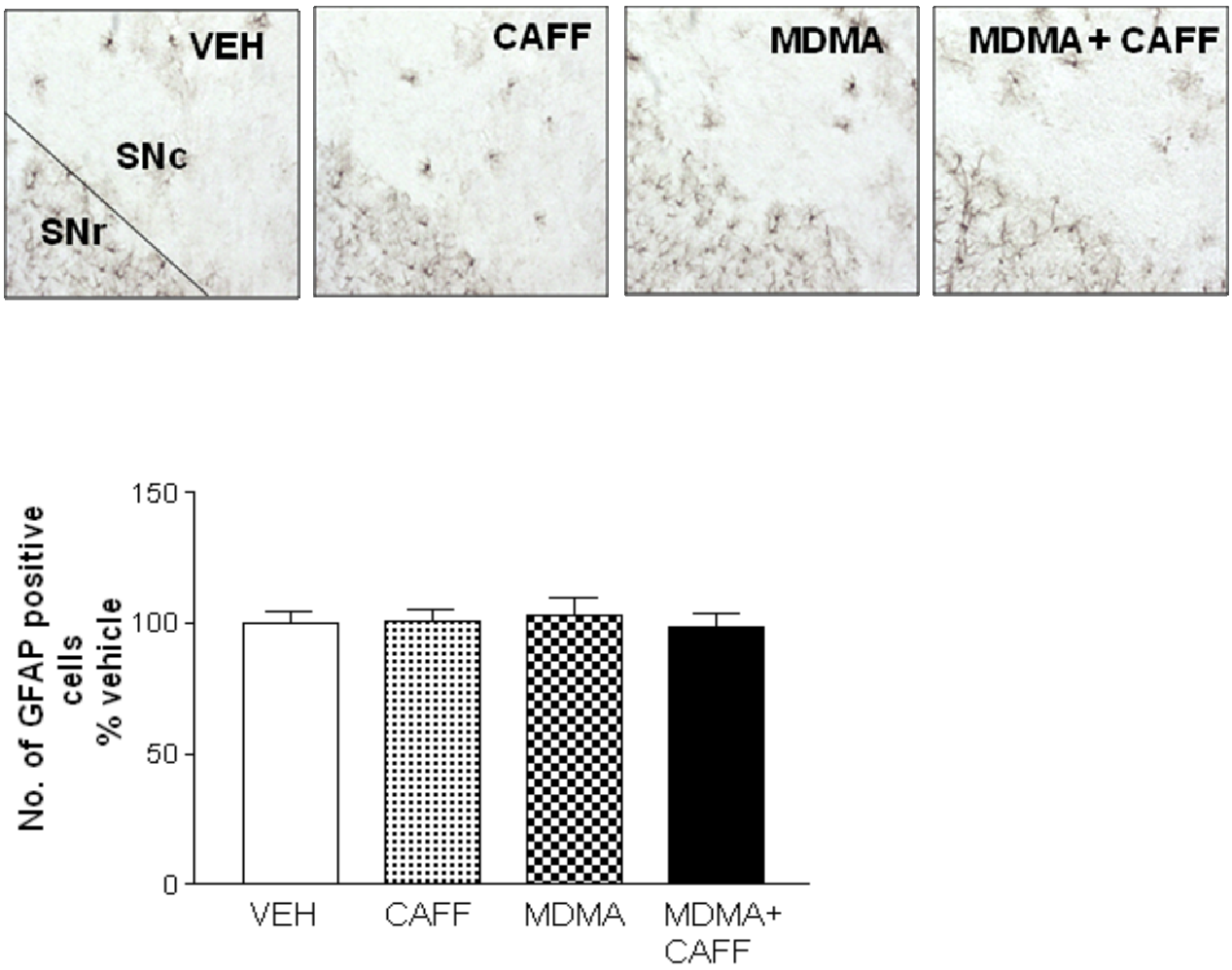


Figure 8.2: Effect of repeated administration of MDMA (20mg/kg, i.p.) and caffeine (10mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker. Representative images and histogram from the substantia nigra pars compacta (SNc) immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM.

Effect of caffeine on MDMA induced CD11b (Microglia) immunoreactivity in striatum

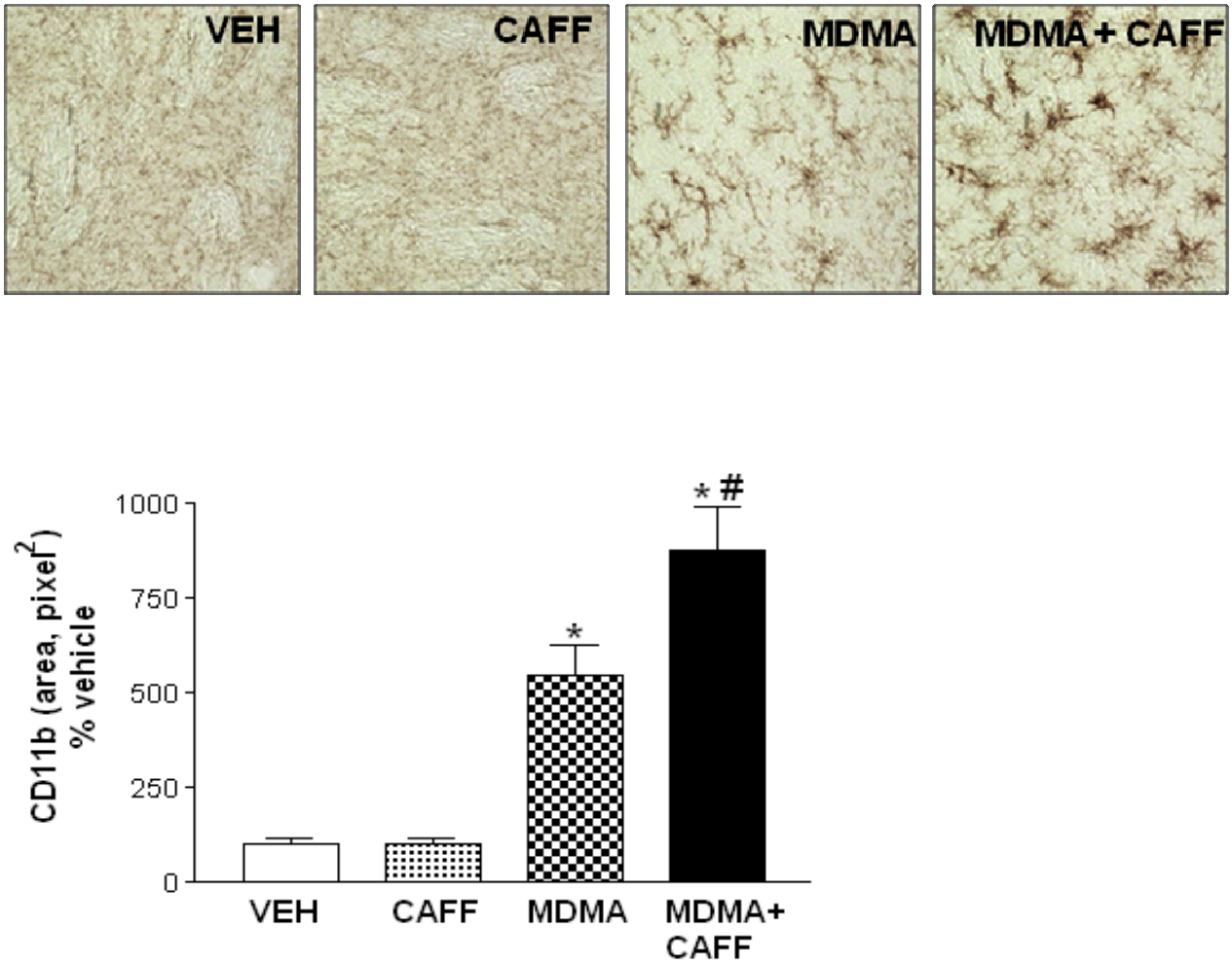
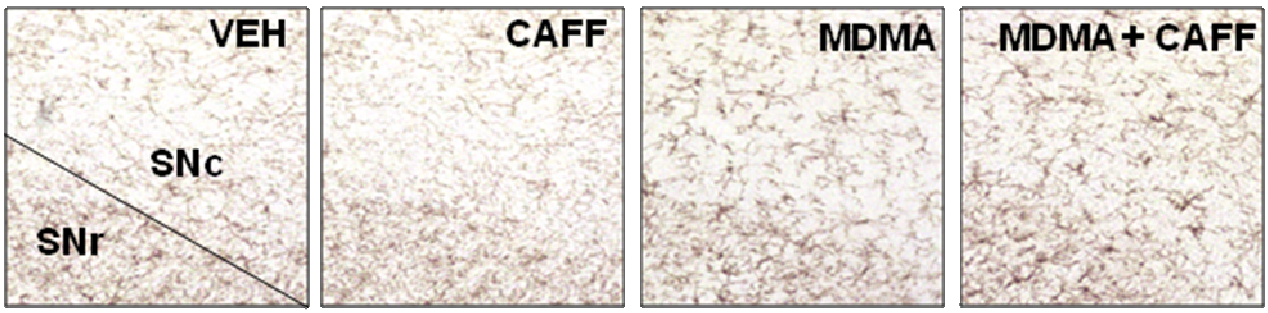


Figure 8.3: Effect of repeated administration of MDMA (20mg/kg ,i.p.) and caffeine (10mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker. Representative images and histogram from the striatum immunostained for CD11b. The area occupied by gray values above a threshold is calculated and expressed as square pixels and as a percentage of staining in vehicle-treated mice and values are expressed as mean ± SEM. *P < 0.05 versus vehicle treated group; $^{\#}$P < 0.05 versus the group treated with MDMA alone.

Effect of caffeine on MDMA induced CD11b (Microglia) immunoreactivity in SNc



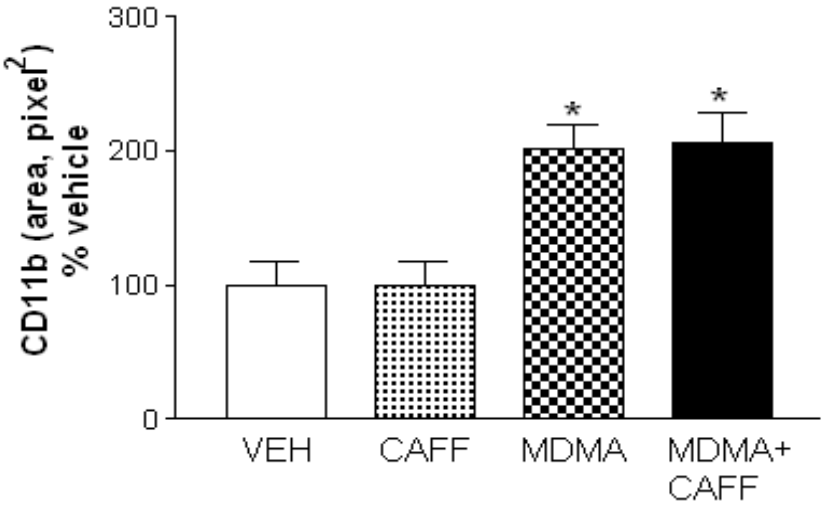


Figure 8.4: Effect of repeated administration of MDMA (20mg/kg, i.p.) and caffeine (10mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker. Representative images and histogram from the SNc immunostained for CD11b. The area occupied by gray values above a threshold is calculated and expressed as square pixels and as a percentage of staining in vehicle-treated mice and values are expressed as mean ± SEM. *P < 0.05 versus vehicle treated group

Effect of caffeine on MDMA induced TH immunoreactivity in SNc

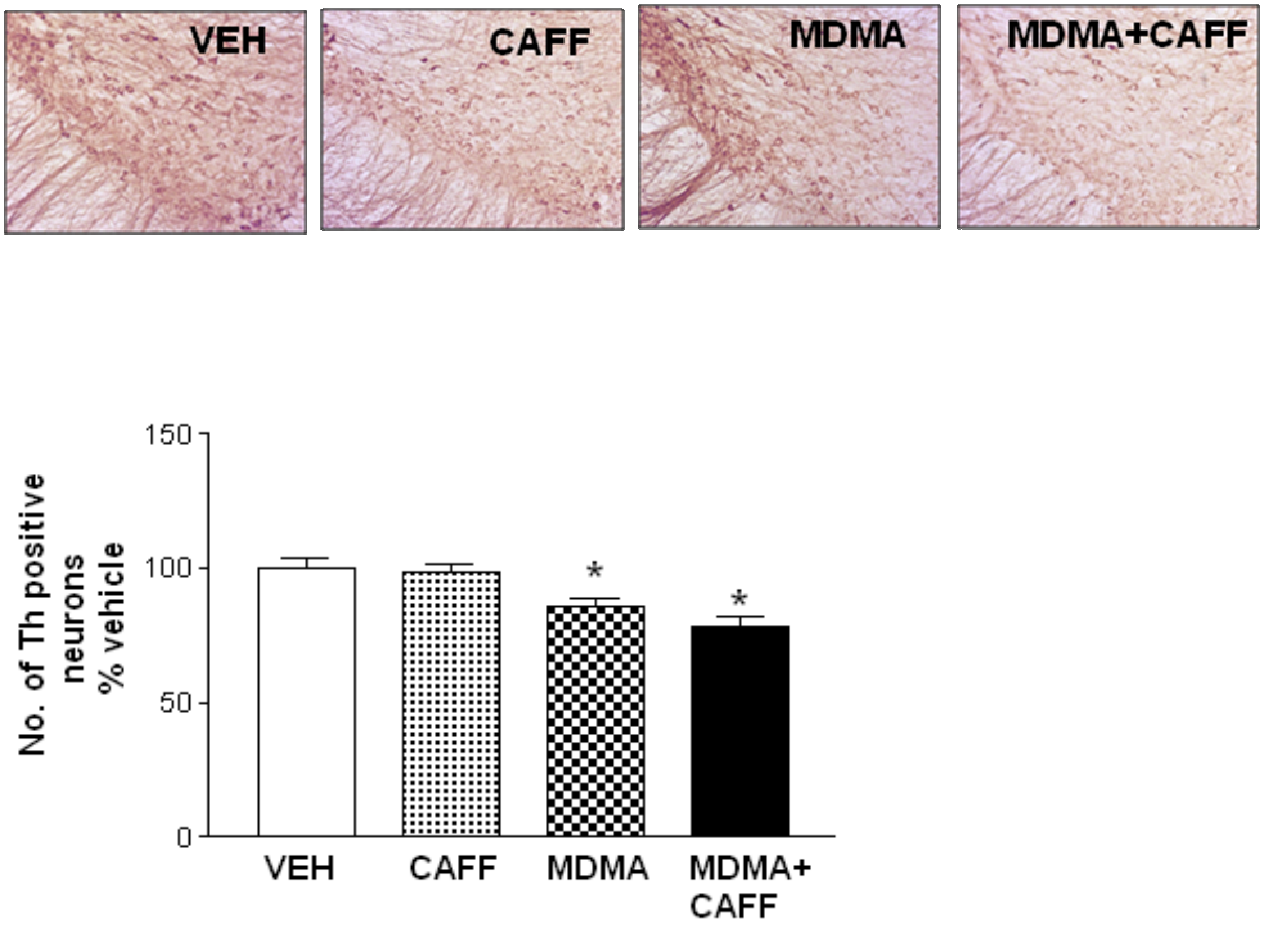
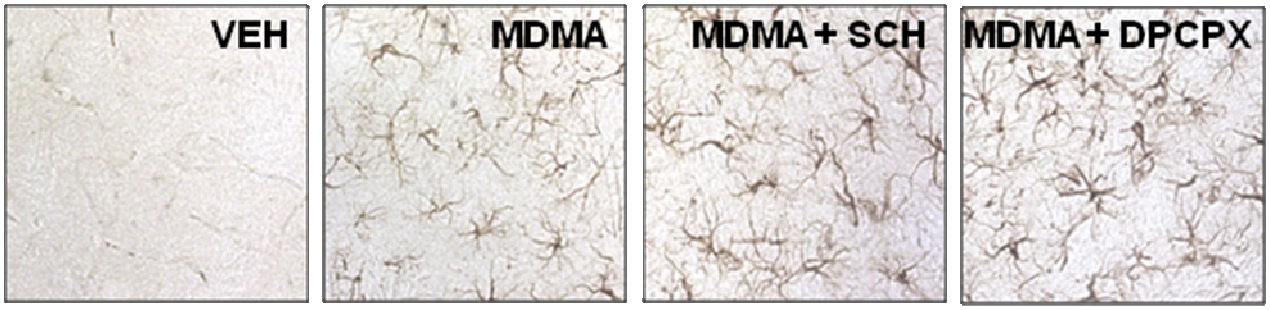


Figure 8.5: Effect of repeated administration of MDMA (20mg/kg, i.p.) and caffeine (10mg/kg, i.p.) on dopaminergic neuronal damage, using TH immunoreactivity as a marker. Representative images and histogram from the SNc immunostained for TH. Values are reported as a percentage of TH immune positive neurons with respect to vehicle treated mice and values are expressed as mean ± SEM. *$P < 0.05$ versus vehicle treated group.

Effect of SCH58261 & DPCPX on MDMA induced GFAP (Astroglia) immunoreactivity in striatum



A) SCH58261 **B) DPCPX**

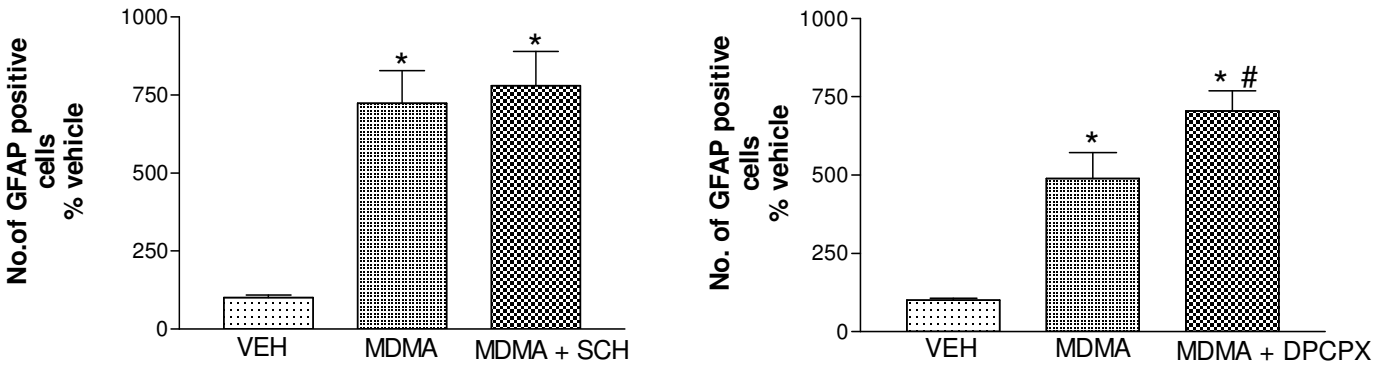


Figure 8.6: Effect of repeated administration of MDMA (20mg/kg, i.p.) and SCH58261 (0.5mg/kg, i.p.), DPCPX (0.5mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker. Representative images and histograms from the striatum immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM. *$P < 0.001$ versus vehicle treated group; $^{\#}P < 0.001$ versus the group treated with MDMA alone.

Effect of SCH58261 & DPCPX on MDMA induced GFAP immunoreactivity in SNc

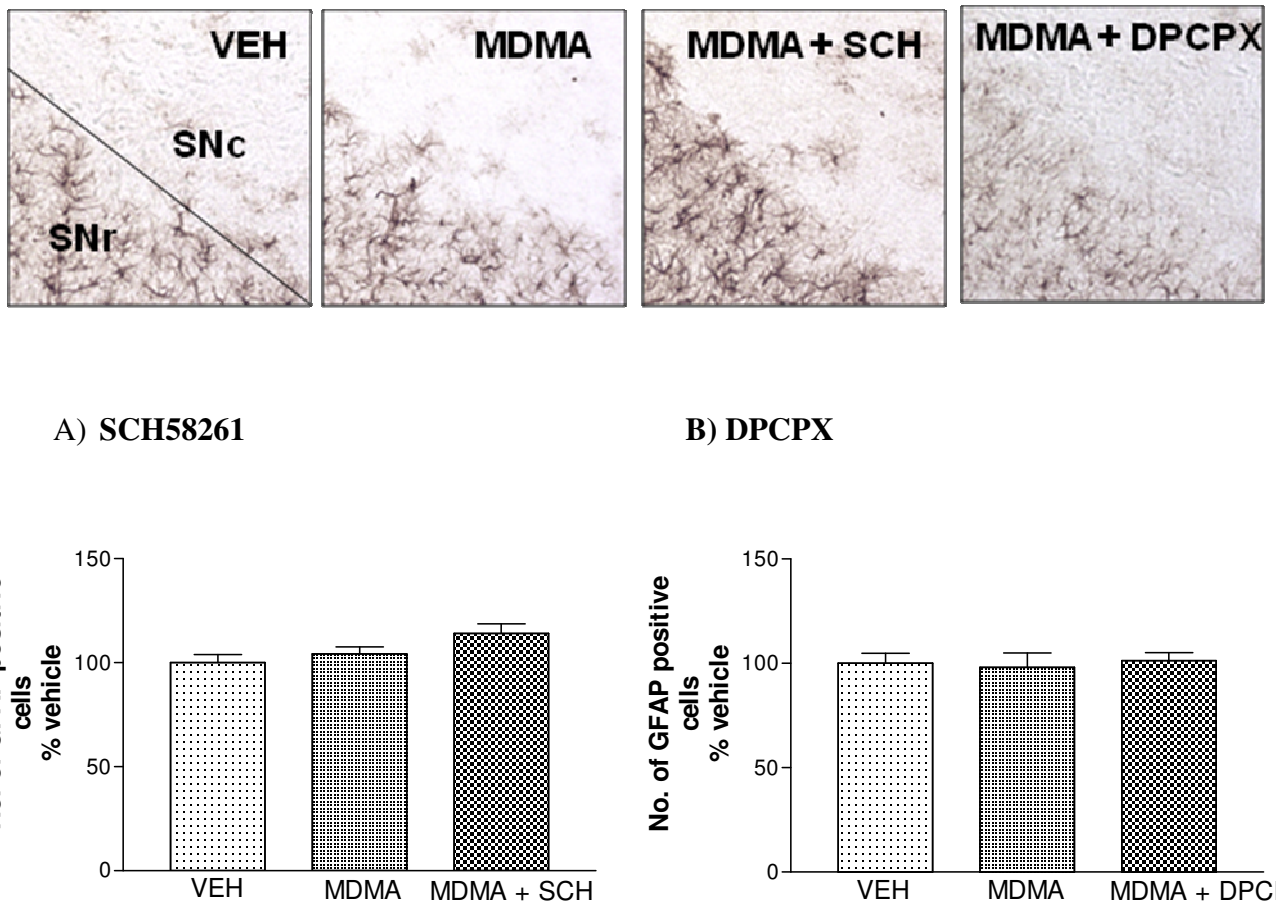
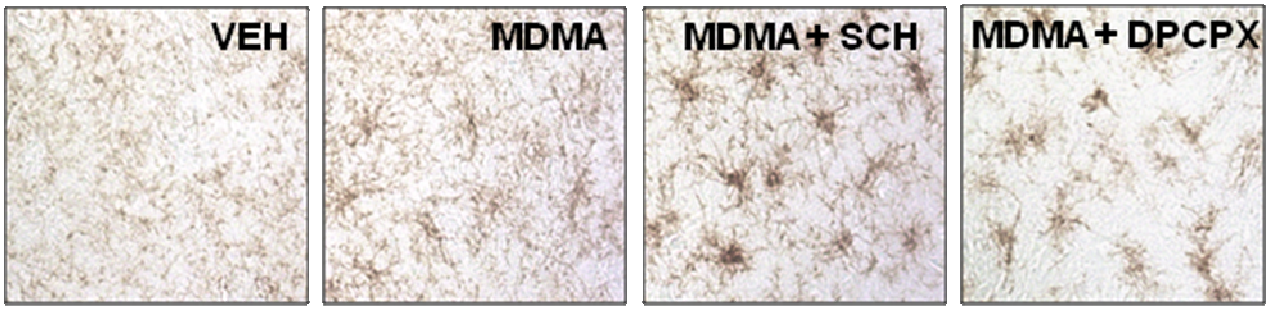
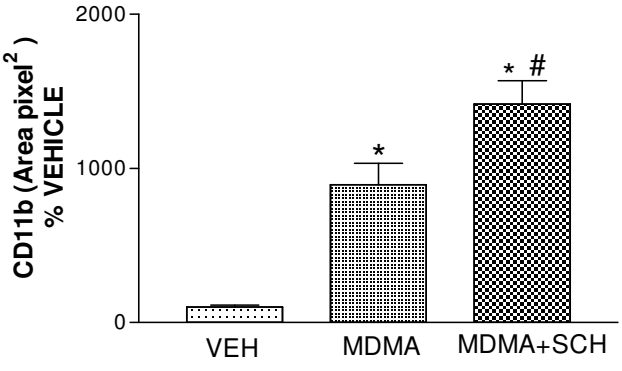


Figure 8.7: Effect of repeated administration of MDMA (20mg/kg,i.p.) and SCH58261 (0.5mg/kg, i.p.), DPCPX (0.5mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker. Representative images and histograms from the SNc immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM.

Effect of SCH58261 & DPCPX on MDMA induced CD11b (Microglia) immunoreactivity in striatum



A) **SCH58261**



B) DPCPX

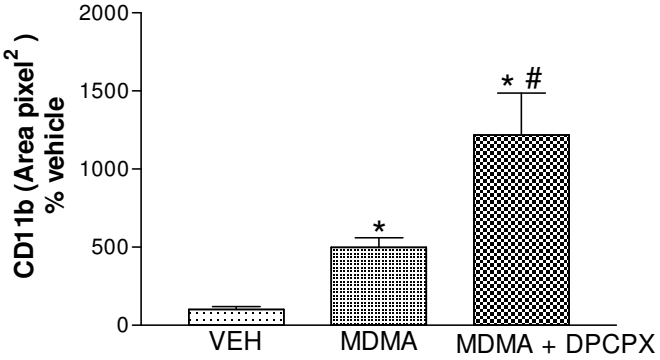
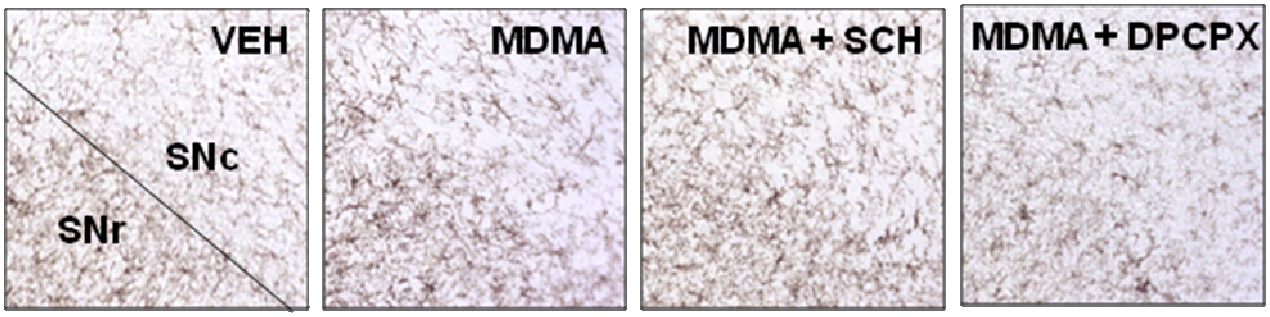


Figure 8.8: Effect of repeated administration of MDMA (20mg/kg, i.p.) and SCH (0.5mg/kg, i.p.), DPCPX (0.5mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker. Representative images and histograms from the striatum immunostained for CD11b. The area occupied by gray values above a threshold is calculated and expressed as square pixels and as a percentage of staining in vehicle-treated mice and values are expressed as mean ± SEM. *$P < 0.05$ versus vehicle treated group; $^{\#}P < 0.05$ versus the group treated with MDMA alone.

Effect of SCH58261 & DPCPX on MDMA induced CD11b (Microglia) immunoreactivity in SNc



A) **SCH58261** **B) DPCPX**

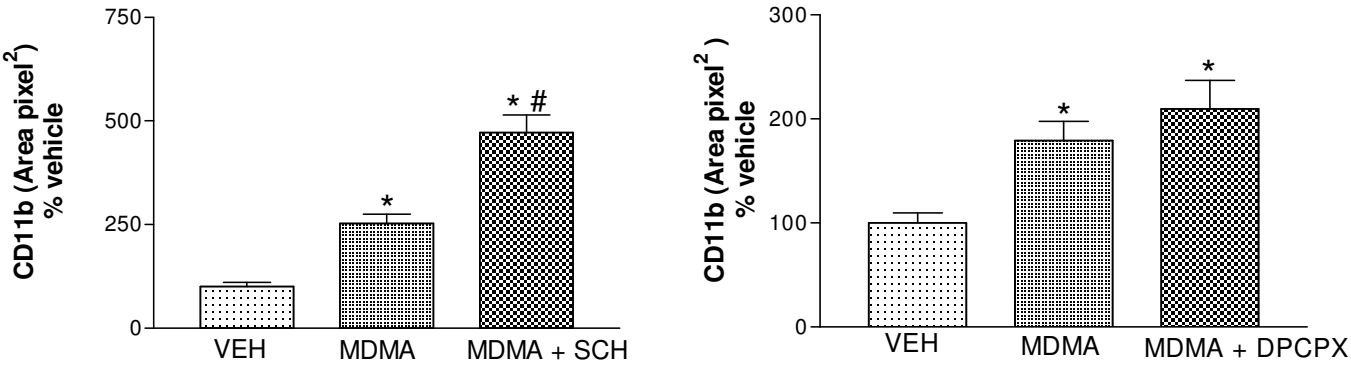


Figure 8.9: Effect of repeated administration of MDMA (20mg/kg, i.p.) and SCH (0.5mg/kg, i.p.), DPCPX (0.5mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker. Representative images and histograms from the striatum immunostained for CD11b. The area occupied by gray values above a threshold is calculated and expressed as square pixels and as a percentage of staining in vehicle-treated mice and values are expressed as mean ± SEM. *$P < 0.05$ versus vehicle treated group; $^{\#}P < 0.05$ versus the group treated with MDMA alone.

Effect of SCH58261 & DPCPX on MDMA induced TH immunoreactivity in SNc

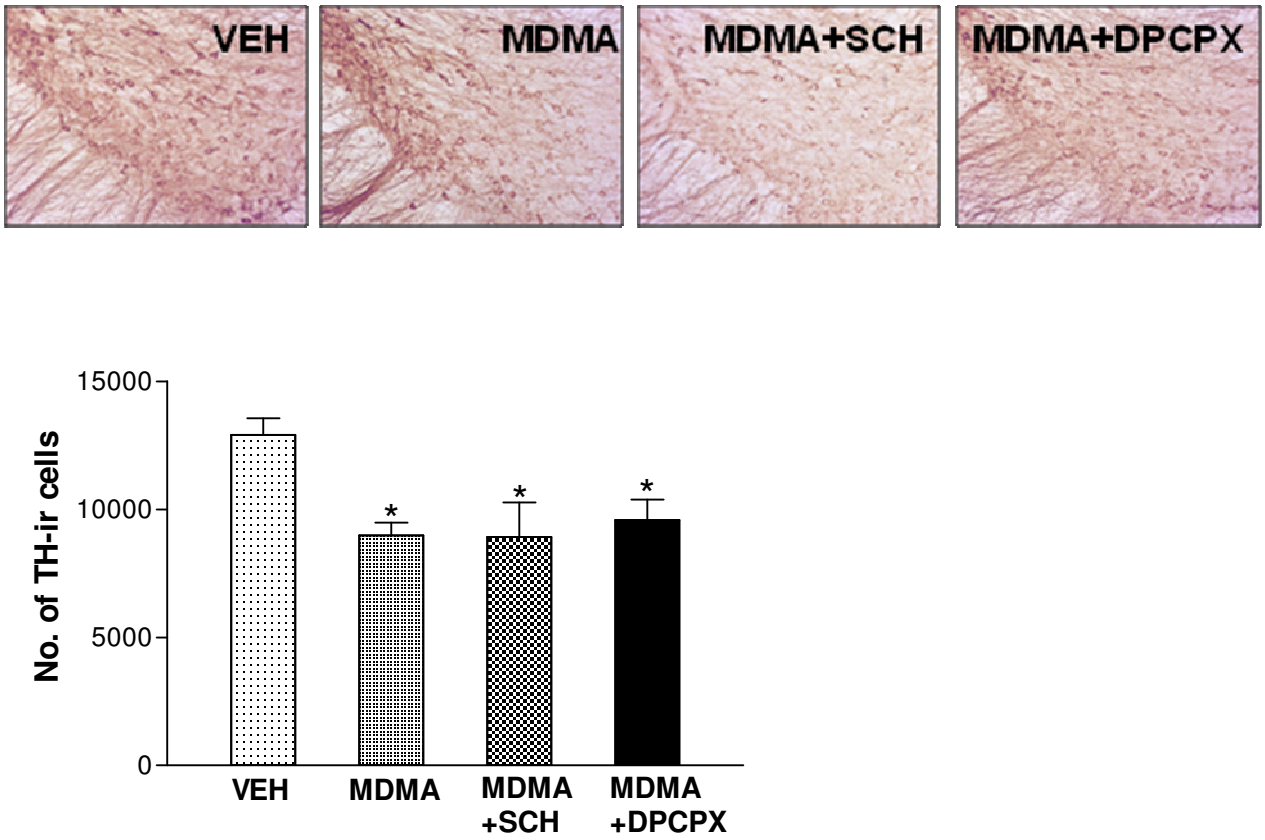


Figure 8.10: Effect of repeated administration of MDMA (20mg/kg,i.p.) and SCH58261 (0.5mg/kg, i.p.), DPCPX (0.5mg/kg, i.p.) on dopaminergic neuronal damage, using TH immunoreactivity as a marker. Representative images and histograms from the SNc immunostained for TH. Values are reported as a percentage of TH immune positive neurons with respect to vehicle treated mice and values are expressed as mean ± SEM. *$P < 0.05$ versus vehicle treated group.

Effect of caffeine on MDMA induced hyperthermia

DRUGS	Basal Temp	1H MDMA	2H MDMA	3H MDMA	4H MDMA
Vehicle	35.525 ± 0.2322	34.875 ± 0.1547	34.675 ± 0.2926	34.525 ± 0.3682	34.90 ± 0.2972
MDMA	35.45 ± 0.2179	36.525 ± 0.3682*	37.125 ± 0.3198*	37.275 ± 0.4607*	36.5 ± 0.2121*
MDMA+CAFF	35.466 ± 0.2458	36.6166 ± 0.2315#	37.55 ± 0.1522#	37.5 ± 0.1549#	37.6333 ± 0.1626#
CAFFEINE	35.575 ± 0.225	35.7 ± 0.1870	35.35 ± 0.3227	35.275 ± 0.3037	35.85 ± 0.1258

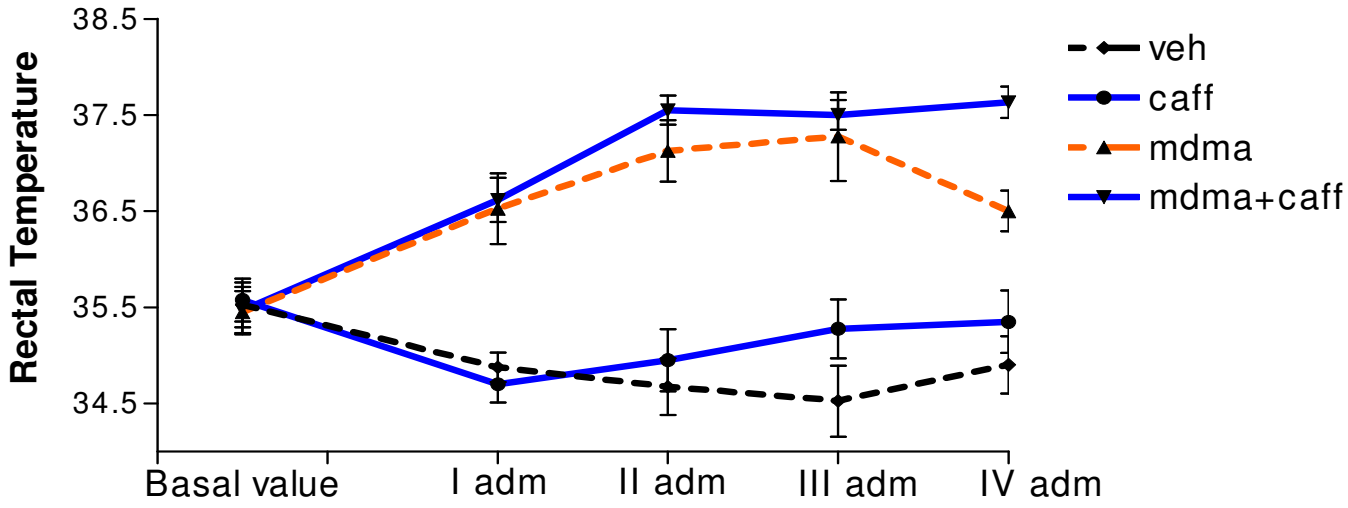


Figure 8.11: Effect of repeated administration of MDMA (4 x 20mg/kg,i.p.) and caffeine (2 x 10mg/kg, i.p.), on MDMA induced hyperthermia every one hour after MDMA administration. *P < 0.05 versus vehicle treated group, #P < 0.05 versus vehicle treated group.

Effect of SCH58261 & DPCPX ON MDMA - induced hyperthermia

DRUGS	Basal Temp	1H MDMA	2H MDMA	3H MDMA	4H MDMA
Vehicle	36.125 ± 0.1108	35.8 ± 0.1870	35.925 ± 0.1376	35.6 ± 0.1914	35.575 ± 0.075
MDMA	36.05 ± 0.1056	37.416 ± 0.0872*	37.7 ± 0.2129*	37.933 ± 0.1333*	37.28 ± 0.2182*
MDMA+SCH58261	36.02 ± 0.1067	37.38 ± 0.1685#	37.76 ± 0.1166#	37.8 ± 0.2073#	37.56 ± 0.1913#
MDMA+DPCPX	36.16 ± 0.1630	37.26 ± 0.16^	37.8 ± 0.1449^	37.84 ± 0.1503^	37.575 ± 0.1648^
SCH58261	35.95 ± 0.35	36.5 ± 0.00	36.1 ± 0.2	36.2 ± 0.1	35.7 ± 0.3
DPCPX	35.9 ± 0.4	35.85 ± 0.15	35.95 ± 0.15	35.8 ± 0.2	35.85 ± 0.45

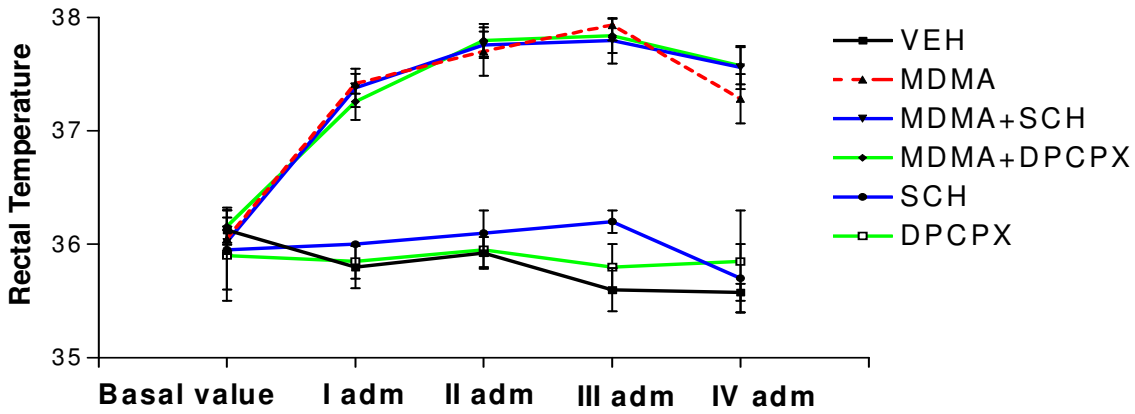


Figure 8.12: Effect of repeated administration of MDMA (4 x 20mg/kg,i.p.) and SCH58261 (2 x 0.5mg/kg, i.p.), DPCPX (0.5mg/kg, i.p.) on MDMA induced hyperthermia every one hour after MDMA administration. *P < 0.05 versus vehicle treated group, #P < 0.05 versus vehicle treated group, ^P < 0.05 versus vehicle treated group.

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Caffeine Enhances Astroglia and Microglia Reactivity Induced by 3,4-Methylenedioxymethamphetamine ('Ecstasy') in Mouse Brain

Amit Khairnar · Antonio Plumitallo · Lucia Frau · Nicoletta Schintu · Micaela Morelli

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Abstract Several reports suggest that 3,4-methylenedioxymethamphetamine (MDMA) induces neurotoxic effects and gliosis. Since recreational use of MDMA is often associated with caffeinated beverages, we investigated whether caffeine interferes with MDMA-induced astroglia and microglia activation, thus facilitating its neurotoxicity. MDMA (4 × 20 mg/kg) was acutely administered to mice alone or in combination with caffeine (10 mg/kg). CD11b and GFAP immunoreactivity were evaluated as markers of microglia and astroglia activation in the substantia nigra pars-compacta (SNc) and striatum. MDMA was associated with significantly higher CD11b and GFAP immunoreactivity in striatum, whereas only CD11b was significantly higher than vehicle in SNc. Caffeine potentiated the increase in CD11b and GFAP in the striatum but not in the SNc of MDMA-treated mice. The abuse of MDMA is a growing worldwide problem; the results of this study suggest that combination of MDMA plus caffeine by increasing glial activation might have harmful health consequences.

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Keywords Glial cell · Adenosine · Drug of abuse · Striatum · Psychostimulants

Introduction

The use of psychostimulant drugs is often combined with beverages containing a high quantity of caffeine in order to amplify their stimulant properties and reduce drowsiness and fatigue. 3,4-Methylenedioxymethamphetamine (MDMA)—known as ecstasy—is one of the most popular psychostimulant whose neurotoxic effects on the central nervous system are often debated. Specific neurotoxic damage to 5-hydroxytryptamine and dopaminergic nerve endings lasting for months in rodents and years in primates has been demonstrated both biochemically and histologically (Green et al. 2003; Granado et al. 2008). These neurotoxic effects appear to result from free radical formation which in turn induces oxidative stress process and from hyperthermia (Green et al. 2003; Goni-Allo et al. 2008).

Evidence for the occurrence of MDMA-induced neurotoxic damage in human users, however, remains ambiguous since such evidence is complicated by the fact that many users often take other substances concomitantly, either intentionally or due to impurities in ecstasy tablets. The most popular of these associated substances is caffeine, which, when given acutely, enhances the acute toxicity and lethality of MDMA (McNamara et al. 2006).

Several findings have suggested that neuroinflammation may play an active role in the pathogenesis of neurodegenerative diseases. Microglial and astroglial activation appear to play an important role in neurotoxicity and MDMA-induced toxicity is associated with microglial activation, which generates many reactive species (e.g., nitric oxide, superoxide, cytokines) favouring neurodegeneration

(Thomas et al. 2004). In this context it becomes of great importance to investigate whether MDMA when given in combination with caffeine, enhances astroglia or microglia reactivity in experimental rodents.

The results of this study may bring important insights to our knowledge of the toxic effects that may be caused by the use of caffeine together with psychostimulant drugs.

Materials and Methods

Drugs

MDMA–HCl (synthesized by Prof. Plumitallo Department of Pharmaceutical Chemistry and Technology, University of Cagliari) was dissolved in saline. Caffeine (Sigma–Aldrich, Milan, Italy) was dissolved in water.

Animals

Adult male C57BL/6J mice, 3 months old (20–25 g; Charles River, Milan, Italy) were maintained at a constant temperature (21 ± 1°C) in 12-h light/dark cycles (lights on at 08:00), and given food and water ad libitum. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the ethics committee of the University of Cagliari.

Treatment

Mice were treated with repeated administration of vehicle ($N = 6$) or MDMA (4 × 20 mg/kg, intraperitoneally, i.p.) ($N = 12$) at 2-h intervals alone or in combination with repeated administration of vehicle or caffeine (2 × 10 mg/kg, i.p.) ($N = 16$) 30 min before the first and third administration of MDMA. On second day mice received two administrations of vehicle or caffeine (10 mg/kg, i.p.) at 12 h interval and one administration on third day. Mice were killed 48 h after the last administration of MDMA. This dosing protocol of MDMA has been shown to produce activation of microglial and astroglial cells (Thomas et al. 2004).

Immunohistochemistry

Animals were anaesthetized with chloral hydrate and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4). Coronal sections (50 μm thick) were cut on a vibratome and immunostained for glial fibrillary acidic protein (GFAP) and CD11b in order to analyze astroglial and microglial activation, respectively. Immunohistochemistry was carried out in free-floating sections with standard avidin–biotin (ABC; Vector Laboratories, UK) immunohistochemical protocols. Sections were treated with hydrogen peroxide to block endogenous peroxidases and then incubated overnight with the following specific primary antisera: monoclonal mouse anti-GFAP (1:400; Sigma–Aldrich) and monoclonal rat anti-CD11b (1:1000; Serotec, UK). For visualization it was used 3,3′-diaminobenzidine as chromogen. Images were captured under constant light conditions using a PixeLink PL-A686 camera at 10× magnification for the SNc and at 20× magnification for the striatum. For each animal, three sections from the SNc ($A = -2.92$, -3.28, -3.64 mm from bregma, accordingly to Mouse brain Atlas by Paxinos and Franklin 2001), and three sections from striatum ($A = 1.10$, 0.74, 0.38 mm from bregma), were analyzed for each protein evaluated in the study.

GFAP immunoreactivity in the SNc was quantified by counting the number of positive cells in the entire left and right part of this structure. For quantification of GFAP immunoreactivity in the striatum, one dorsolateral and one ventromedial portion were analyzed from both the left and right parts using PixeLink image analysis software. Analysis of CD11b immunoreactivity in the SNc and striatum was done with the analysis software Scion Image. Within each frame, the area occupied by gray values above the threshold was automatically calculated.

In order to obtain averages for GFAP and CD11b, values were normalized with respect to vehicle.

All values were expressed as mean ± SEM and were statistically analyzed with a one-way ANOVA followed by the Newman–Keuls post hoc test.

Results

Previous studies have shown that expression of CD11b and GFAP, markers of activated microglia and astroglia, respectively, reached their maximum at 48 and 72 h after MDMA administration (Granado et al. 2008; Thomas et al. 2004). Therefore, in our study, mice were killed 48 h after last administration of MDMA in order to examine the activation of both microglia and astroglia.

GFAP Immunoreactivity in the Striatum and SNc

GFAP immunoreactivity was low in both the striatum and SNc in the basal condition (Fig. 1). It was significantly higher in the striatum after treatment with MDMA (20 mg/kg, i.p.) and MDMA plus caffeine (10 mg/kg, i.p.) compared with vehicle ($P < 0.0001$). Post hoc analysis indicated that administration of caffeine in MDMA-treated mice was associated with significantly higher GFAP immunoreactivity in the striatum (Fig. 1a) compared with

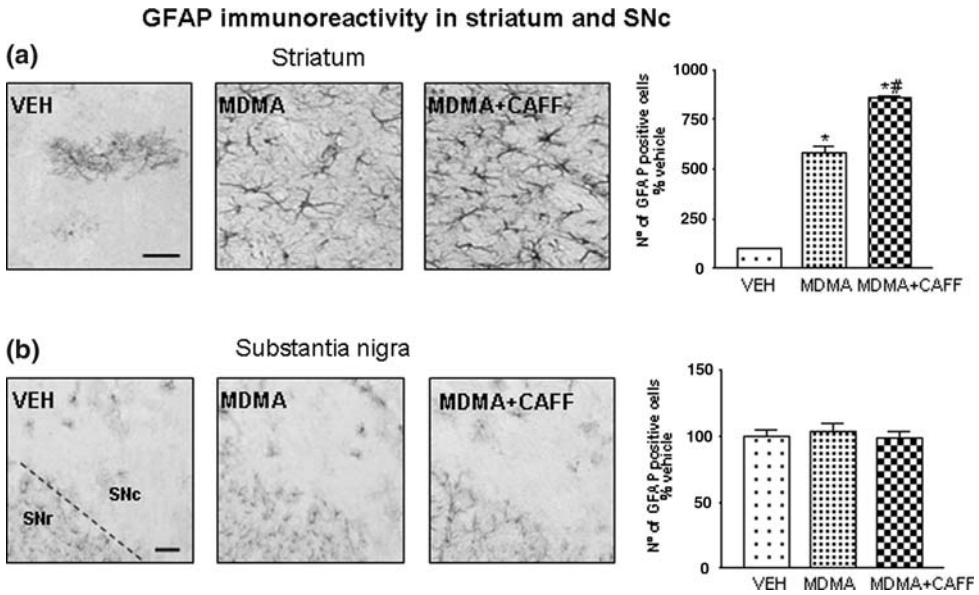

Fig. 1 Effect of repeated administration of MDMA (20 mg/kg, i.p.) and caffeine (10 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker. Representative images and histograms from the **a** striatum and **b** SNc, immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM. * $P < 0.0001$ versus vehicle-treated group; # $P < 0.0001$ versus the group treated with MDMA alone. *Scale bar*: 50 μm. *SNc* substantia nigra pars-compacta, *SNr* substantia nigra pars-reticulata

mice treated only with MDMA ($P < 0.0001$). Treatment with MDMA and MDMA plus caffeine did not alter GFAP immunoreactivity in the SNc compared with vehicle (Fig. 1b). Caffeine alone did not modify GFAP both in striatum and SNc (data not shown).

CD11b Immunoreactivity in the Striatum and SNc

Similarly, to GFAP, CD11b immunoreactivity was low in both the striatum and SNc in the basal condition (Fig. 2). It was significantly higher in both the striatum (Fig. 2a) and

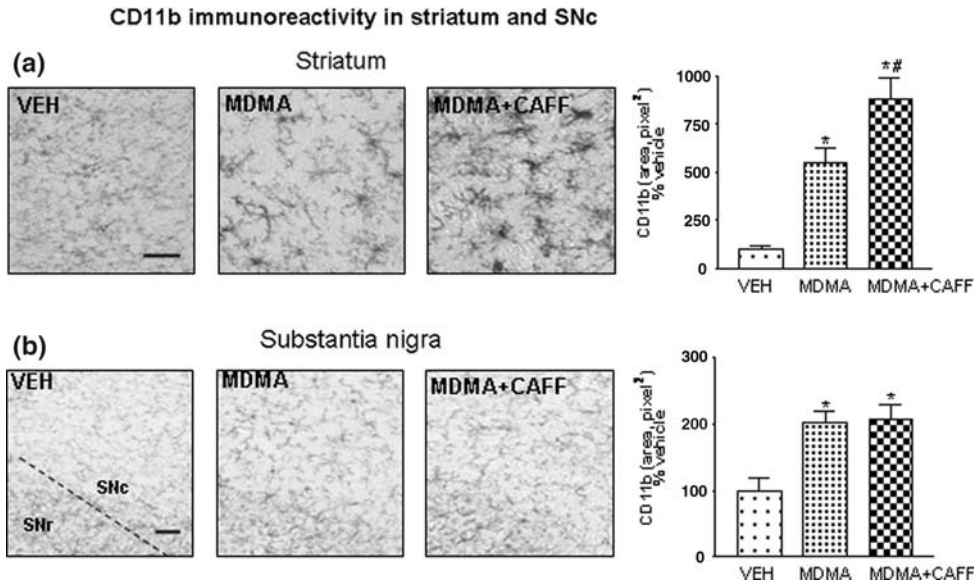

Fig. 2 Effect of repeated administration of MDMA (20 mg/kg, i.p.) and caffeine (10 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker. Representative images and histograms from the **a** striatum and **b** SNc, immunostained for CD11b. The area occupied by *gray* values above a threshold was calculated and expressed as *square pixels* and as a percentage of staining in vehicle-treated mice, and values are expressed as mean ± SEM. * $P < 0.02$ versus vehicle-treated group, # $P < 0.05$ versus the group treated with MDMA alone. *Scale bar*: 50 μm. *SNc* substantia nigra pars-compacta, *SNr* substantia nigra pars-reticulata

SNc (Fig. 2b) after treatment with MDMA (20 mg/kg, i.p.) and MDMA plus caffeine (10 mg/kg, i.p.) compared with vehicle ($P < 0.05$). Post hoc analysis indicated that administration of caffeine in MDMA-treated mice was associated with significantly higher CD11b immunoreactivity in the striatum but not in the SNc compared with mice treated only with MDMA ($P < 0.05$). Caffeine alone did not modify CD11b levels both in striatum and SNc (data not shown).

Discussion

The results of the present study show that acute repeated administration of MDMA induced a neuroinflammatory process in mice, characterized by microgliosis in the striatum and SNc and astrogliosis in the striatum. Moreover, of great importance in terms of possible consequences in humans is the finding that caffeine, when given together with MDMA, potentiated the activation of microglia and astroglia in the striatum.

Caffeine is a safe substance present in beverages such as coffee, tea, soft drinks, and energy drinks. However, as shown by the present study, caffeine taken with MDMA at doses similar to those that may be taken for recreational use with energy drinks may exacerbate MDMA toxicity posing health consequences for consumers of this drugs of abuse.

Neuroinflammation is one of the factors responsible for the pathogenesis of neurodegenerative disorders (Kerschensteiner et al. 2009). Studies have reported that microglial and astroglial activation represent an early step in MDMA-induced neurotoxicity and that within the amphetamine class of drugs only those causing neurotoxicity result in microglia activation (Thomas et al. 2004; Granado et al. 2008).

Moreover, it is well-known that activation of glial cells, particularly microglia, may contribute to neuronal damage by the release of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and neurotoxic factors that include reactive nitrogen species, reactive oxygen species, and excitatory amino acids (Kerschensteiner et al. 2009), although a neuroprotective role of glial cells has also been hypothesized (Merrill and Benveniste 1996). It has also been shown that proinflammatory molecules such as neuronal nitric oxide synthase (NOS) and inducible NOS are increased 1 day after MDMA administration and that their inhibition provides protection against MDMA-induced dopamine loss in the striatum (Green et al. 2003). Therefore, by showing higher activation of both microglia and astroglia after MDMA plus caffeine compared with MDMA alone, our results suggest that this drug combination may facilitate the neurodegenerative processes induced by MDMA in mouse brain. Interestingly, studies on MPTP model of Parkinson's disease have shown that striatal dopaminergic terminals are more vulnerable than SNc cell bodies (Schmidt and Ferger 2001), being in line with the higher glial activation in striatum observed in our study.

One of the mechanisms implicated in MDMA neurotoxicity is a hyperthermic response induced by the drug. Consistent hyperthermia has been observed in rats and a hyperthermic response, though much more variable, has been seen in mice (Green et al. 2003; McNamara et al. 2006). Moreover, promotion of temperature elevation by caffeine was observed (McNamara et al. 2006), suggesting a possible mechanism that may potentiate MDMA effects, contributing to the increase in astroglia and microglia reactivity observed after MDMA plus caffeine administration.

Caffeine affects the absorption of MDMA in intestinal epithelial cells and increases the area under the plasma concentration curve of MDMA (Kuwayama et al. 2007). This effect, as well as hyperthermia, may have a role in caffeine's potentiation of MDMA effects (Green et al. 2003; Goni-Allo et al. 2008). A further consideration that should be taken into account is that caffeine and MDMA are extensively metabolized, and caffeine metabolites have a long half-life. Therefore, the possibility that their metabolites may have a role in glial activation cannot be ruled out. Interestingly, however, it has been established that caffeine does not inhibit CYP2D6, the enzyme responsible for MDMA metabolism, and it did not alter the half maximal inhibitory concentration for MDMA (Downey and O'Boyle 2007).

The results of the present study might seem to contradict the results of studies showing that caffeine counteracts other forms of toxicity. On the other hand, several studies have shown that the role of adenosine can be neuroprotective or neurotoxic depending on the type of insult and the specific cellular conditions (Cunha 2001). Therefore, it is reasonable to hypothesize that endogenous adenosine may counteract MDMA-induced glial activation. Adenosine may in fact decrease blood pressure, heart rate and induce hypothermia (Jonzon et al. 1986) as well as regulate the actions of neurotrophic factors through adenosine A_{2A} receptors (Sebastião and Ribeiro 2009). By blocking both adenosine A_1 and A_{2A} receptors, caffeine may facilitate neuroinflammatory processes induced by MDMA. It should be, however, emphasized that our study relates to acute caffeine treatment and that due to the rapid tolerance developed to the drug, different results might be obtained in a chronic protocol of caffeine administration.

In conclusion, by activating astroglia and microglia cells, the combination of MDMA plus caffeine enhanced inflammatory brain processes induced by MDMA. Since MDMA and energy drinks are very popular among young

people, particular attention should be paid in the harmful health consequences of this combination of drugs.

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Neuroprotective and Anti-inflammatory Effects of the Adenosine A_{2A} Receptor Antagonist ST1535 in a MPTP Mouse Model of Parkinson's Disease

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KEY WORDS TH; dopamine; neuron degeneration; CD11b; GFAP; striatum; substantia nigra compacta

ABSTRACT Adenosine A_{2A} receptor antagonists are one of the most attractive classes of drug for the treatment of Parkinson's disease (PD) as they are effective in counteracting motor dysfunctions and display neuroprotective and anti-inflammatory effects in animal models of PD. In this study, we evaluated the neuroprotective and anti-inflammatory properties of the adenosine A_{2A} receptor antagonist ST1535 in a subchronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. C57BL/6J mice were repeatedly administered with vehicle, MPTP (20 mg/kg), or MPTP + ST1535 (2 mg/kg). Mice were sacrificed three days after the last administration of MPTP. Immunohistochemistry for tyrosine hydroxylase (TH) and cresyl violet staining were employed to evaluate dopaminergic neuron degeneration in the substantia nigra pars compacta (SNc) and caudate-putamen (CPu). CD11b and glial fibrillary acidic protein (GFAP) immunoreactivity were, respectively, evaluated as markers of microglial and astroglial response in the SNc and CPu. Stereological analysis for TH revealed a 32% loss of dopaminergic neurons in the SNc after repeated MPTP administration, which was completely prevented by ST1535 coadministration. Similarly, CPu decrease in TH (25%) was prevented by ST1535. MPTP treatment induced an intense gliosis in both the SNc and CPu. ST1535 totally prevented CD11b immunoreactivity in both analyzed areas, but only partially blocked GFAP increase in the SNc and CPu. A_{2A} receptor antagonism is a new opportunity for improving symptomatic PD treatment. With its neuroprotective effect on dopaminergic neuron toxicity induced by MPTP and its antagonism on glial activation, ST1535 represents a new prospect for a disease-modifying drug. **Synapse 00:000–000, 2010.**

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor dysfunctions because of a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Causes of this neurodegeneration are not clear, but in the last decade evidence has linked it to intense neuroinflammation (Hirsch et al., 2003; McGeer and McGeer, 2008; Rogers et al., 2007; Tansey et al., 2007; Teismann et al., 2003; Whitton, 2007). In such inflammatory states, activated glia can produce large quantities of free radicals, cytokines, and other neurotoxic substances to which dopaminergic neurons appear very sensitive and which could be related with the progression of the disease (Hirsch et al., 1998; Hunot and Hirsch, 2003; McGeer et al., 2001).

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One of the main targets of research in PD is to identify new molecules that are not only able to ameliorate motor symptoms but also protect dopaminergic neurons and halt the progression of their degeneration. Among the new therapies proposed, adenosine A_{2A} receptor antagonists are the best candidates, since preclinical studies and clinical trials suggest that these compounds may increase the therapeutic efficacy of L-3,4-dihydroxyphenylalanine (L-DOPA) without exacerbating L-DOPA-associated dyskinetic effects (Bara-Jimenez et al., 2003; Grondin et al., 1999; Hauser et al., 2008; Kanda et al., 2000; Lewitt et al., 2008; Morelli, 2003; Schwarzschild et al., 2006; Stacy et al., 2008). Furthermore, epidemiological studies have shown that the incidence of PD is lower in consumers of high doses of caffeine, an antagonist of adenosine A_1 and A_{2A} receptors (Ross et al., 2000). A_{2A} receptor antagonists have also been shown to have a neuroprotective role in several experimental rodent models of PD (Carta et al., 2009; Ikeda et al., 2002; Pierri et al., 2005). In the case of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, similar neuroprotection was obtained using A_{2A} receptor knockout mice (Carta et al., 2009). Interestingly, A_{2A} receptors are present in the basal ganglia, particularly in the caudate-putamen (CPu), where they are localized postsynaptically in GABAergic striatopallidal neurons and presynaptically in cortical nerve terminals (Ongini and Fredholm, 1996; Rebola et al., 2005; Rosin et al., 1998; Schiffmann et al., 2007; Svenningsson et al., 1999). Moreover, A_{2A} receptors are expressed in non-neuronal cell types, such as microglia and astroglia (Fiebich et al., 1996; Saura et al., 2005).

Previous studies have shown that the non-xanthine compound 2-butyl-9-methyl-8-(2H-1,2,3-triazol-2-yl)-9H-purin-6-ylamine (ST1535) has antiparkinson activity since it blocks catalepsy induced by the adenosine A_{2A} receptor agonist CGS 21680 and by haloperidol (Minetti et al., 2005; Stasi et al., 2006), and produces positive effects in acute models of parkinsonian akinesia and tremor in rats (Pinna et al., 2007) and monkeys (Rose et al., 2006). Moreover, when administered subchronically in unilaterally 6-hydroxydopamine (6-OHDA) lesioned rats with a threshold dose of L-DOPA, ST1535 induced contralateral turning at a similar intensity to that produced by a full dose of L-DOPA without exacerbating its dyskinetic effects (Tronci et al., 2007). In addition, ST1535 reduces long-term neuronal modifications correlated to L-DOPA motor side effects, as induction of the early gene *zif-268* mRNA (Tronci et al., 2007).

ST1535 also modulates corticostriatal glutamatergic transmission (Tozzi et al., 2007), reduces striatal extracellular glutamate levels (Galluzzo et al., 2008), and protects the striatum against rotenone-induced neurotoxicity (Belcastro et al., 2009).

Given the positive symptomatic preclinical studies, the aim of the present study was to evaluate the neuroprotective and anti-inflammatory effects of ST1535 in a subchronic MPTP mouse model of PD. Neuron damage and the neuroprotective effect of ST1535 were evaluated by tyrosine hydroxylase (TH) immunoreactivity and cresyl violet staining in the SNc, whereas the antiinflammatory efficacy of ST1535 was evaluated in the SNc and CPu by immunohistochemistry for CD11b and glial fibrillary acidic protein (GFAP), as markers of microglia and astroglia, respectively. Doses of ST1535 in this study were chosen on the basis of previous studies performed with the prototypical A_{2A} receptor antagonist SCH58261, which showed that ST1535 has a potency of about four times lower than SCH58261 (Pinna et al., 2007, 2010).

Abbreviations:

6-OHDA	6-hydroxydopamine
CPu	caudate-putamen
DMSO	dimethyl sulfoxide
GFAP	glial fibrillary acidic protein
L-DOPA	L-3,4-dihydroxyphenylalanine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
PEG	polyethylene glycol
ROI	region of interest
SNc	substantia nigra pars compacta
TH	tyrosine hydroxylase

MATERIALS AND METHODS

Drugs

The 2-butyl-9-methyl-8-(2H-1,2,3-triazol-2-yl)-9H-purin-6-ylamine (ST1535) (Sigma-Tau, Italy) was dissolved in 5% dimethyl sulfoxide (DMSO), 45% polyethylene glycol 400 (PEG 400), and 50% water. MPTP-HCl (Sigma, Italy and USA) was dissolved in water.

Treatments

Male C57BL/6J mice, 3 months old (Charles River, Italy), were treated with vehicle (5% DMSO, 45% PEG 400, and 50% water) (n = 4–5 for TH analysis, n = 13–14 for CD11b and GFAP analysis); vehicle + MPTP (20 mg/kg, i.p.) (n = 5 for TH analysis, n = 13–16 for CD11b and GFAP analysis); or ST1535 (2 mg/kg, i.p.) + MPTP (20 mg/kg, i.p.) (n = 5 for TH analysis, n = 13–16 for CD11b and GFAP analysis). Mice received four doses of MPTP, administered once a day for four days. During MPTP treatment, mice received ST1535 or vehicle twice a day, 30 minutes before and 12 hours after MPTP administration. Animals also received ST1535 or vehicle the day before MPTP treatment once a day, and after MPTP treatment discontinuation, once a day, until sacrifice, which occurred three days after the last administration of MPTP. Mice were anesthetized with chloral

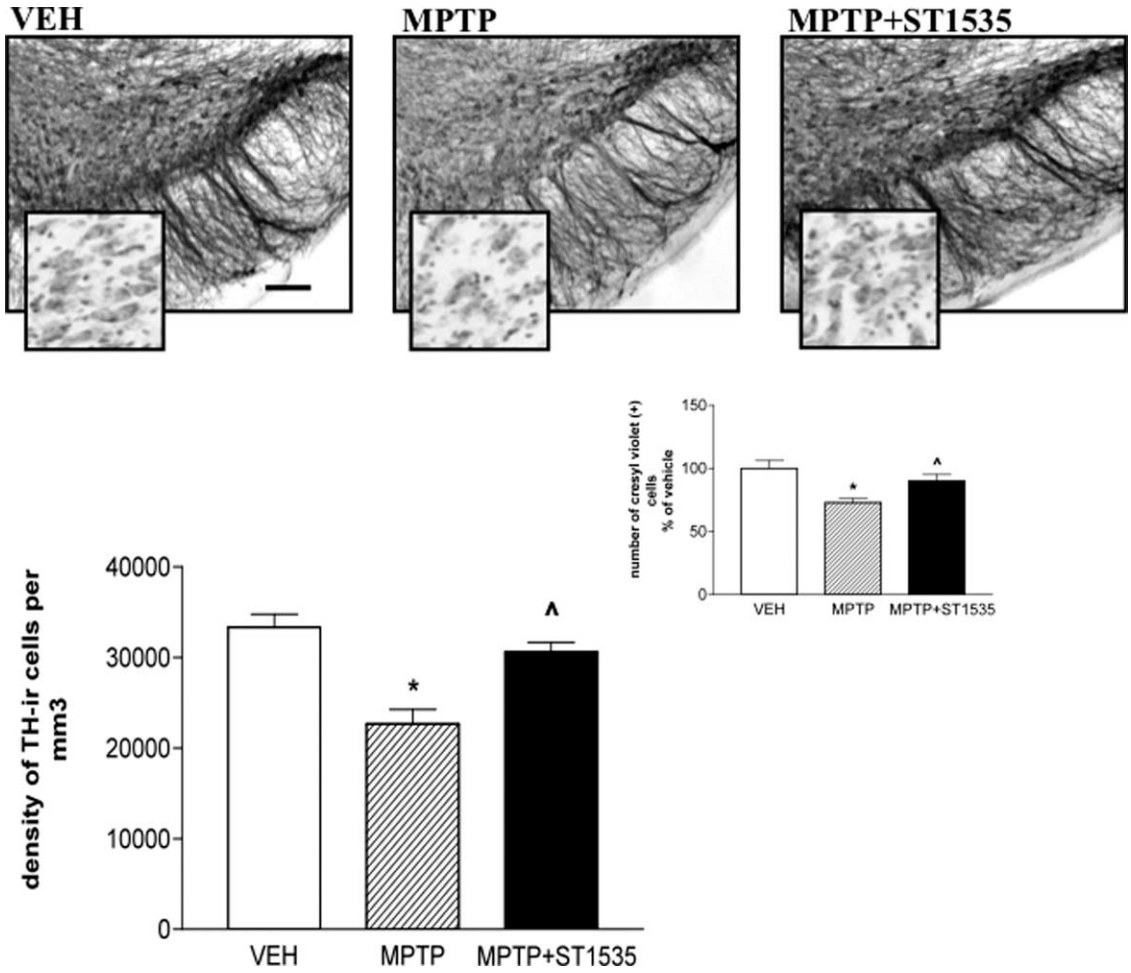

Fig. 1. Immunoreactivity for TH and cresyl violet staining in SNc. Representative coronal sections of SNc immunostained for TH and stained for cresyl violet (see inserts). Mice were treated with MPTP (20 mg/kg once a day for four days, i.p.) plus vehicle or ST1535 (2 mg/kg, i.p. twice a day during MPTP treatment, once a day the day before and two days after MPTP) and sacrificed three days after MPTP treatment. The graph shows the density of TH-immunoreactive cells per mm^3 expressed as a percentage with respect to vehicle-treated mice. The graph on top right shows the number of cresyl violet-positive cells expressed as a percentage with respect to vehicle-treated mice. Values are expressed as mean ± SEM. $^*P < 0.001$ as compared to the vehicle-treated mice; $\hat{}P < 0.001$ as compared to the MPTP-treated mice, by Tukey's post hoc test. $n = 4–5$. Scale bar, 50 μm.

hydrate (400 mg/kg, i.p.) and transcardially perfused with paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4) for immunohistochemistry.

Immunohistochemistry and cresyl violet staining

Sections from the SNc and CPu (50-μm thick) were coronally cut on a vibratome and immunoreacted with TH, CD11b, and GFAP antibodies (polyclonal rabbit anti-TH, 1:1000, Biomol, UK; monoclonal rat anti-mouse CD11b, 1:1000, Serotec, UK; monoclonal mouse anti-GFAP, 1:400, Sigma, Italy), and proper secondary antibodies (goat anti-rabbit immunoglobulin G (IgG) for TH, goat anti-rat IgG for CD11b, and goat anti-mouse IgG for GFAP, all from Vector, UK). For visualization, the avidin/biotin-peroxidase protocol (ABC, Vector, UK) was applied, using 3,3′-diaminobenzidine (Sigma, Italy) as chromogen. Sections were mounted on gelatin-coated slides, dehydrated, and cover slipped (Schintu et al., 2009a,b).

Adjacent SNc sections were stained with cresyl violet to evaluate cell death in this area. Images were digitized (PL-A686 video camera, Pixelink, Canada) under constant light conditions. In each section, the entire left and right SNc were analyzed, whereas for the CPu evaluation, one portion from the dorsolateral CPu and one from the ventromedial CPu (520 μm × 380 μm), left and right, were analyzed. For each animal, three sections from the SNc ($A = -2.92$ mm, -3.28 mm, -3.64 mm from bregma, according to the mouse brain atlas by Paxinos and Franklin (2001)) and three sections from the CPu ($A = 1.10$ mm, 0.74 mm, and 0.38 mm from bregma) were analyzed for

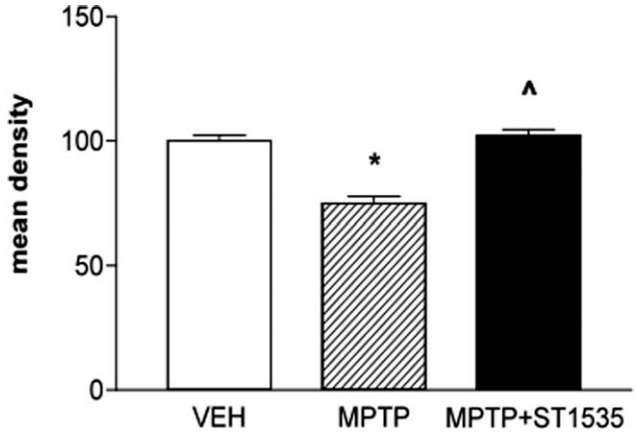

Fig. 2. Immunoreactivity for TH in CPu. Mice were treated with MPTP (20 mg/kg once a day for four days, i.p.) plus vehicle or ST1535 (2 mg/kg, i.p. twice a day during MPTP treatment, once a day the day before and two days after MPTP) and sacrificed three days after MPTP treatment. The graph shows the mean density of gray value of TH expressed as a percentage with respect to vehicle-treated mice. Values are expressed as mean ± SEM. $^*P < 0.001$ as compared to the vehicle-treated mice; $^\wedge P < 0.001$ as compared to the MPTP-treated mice, by Tukey's post hoc test. $n = 5$–6.

each protein evaluated in the study and for cresyl-violet-stained cells.

Stereological counting of TH-immunoreactive neurons

TH-immunoreactive neurons were counted on both hemispheres. All stereological counting was performed using a Leica microscope (DMLB; Leica, Denmark) equipped with a camera (Basler Vision Technologies, Germany) and a stage connected to an *xyz* stepper (PRIOR ProScan) and the newCAST Visiopharm (Denmark) software. The SNc region was outlined at low magnification (5×) for area estimation. The number of labeled neurons was calculated under 63× magnification using randomized meander sampling and optical dissector methods. The cut thickness of sections was 50 μm and the optical dissector height was 12 μm. The top (13 μm) and bottom (25 μm) layers that shrunk during staining procedure were discarded. The sampling area covered 100% of the region of interest (ROI). The counting frame (8302,8 μm^2) applied the exclusion and inclusion lines, and unbiased counting was performed by an experimenter blinded to the treatment. Results are presented, according to Carta et al. (2009), as the mean of TH-immunoreactive neurons per mm^3 ±SEM, calculated using the following formula:

$$D = \Sigma Q / V \text{ sampling}$$

where D = density of labeled cells per mm^3, V sampling = area of the region of interest × dissector height, Q = total count of labeled neurons.

In addition, we have extrapolated the numbers of dopaminergic neurons in whole SNc using the following formula:

$$N = D \times V$$

where D = density of cells per mm^3, V = volume of the SNc.

The volume of SNc was reconstructed by calculating the distance among the three slices, multiplied by the outlined area.

Analysis of TH immunoreactivity in the CPu

Images were digitized in gray scale, and TH immunoreactivity analysis in the CPu was performed using the image analysis program Scion Image (Scion Corp., USA). The average gray values from white matter were subtracted from each section to correct for background immunoreactivity. For each level of CPu, the obtained value was first normalized with respect to vehicle, and values from different levels were averaged thereafter.

Analysis of GFAP immunoreactivity

For each SNc and CPu level, the mean number of GFAP-positive cells obtained from each experimental group was first normalized with respect to vehicle, and values from different levels were averaged thereafter.

Analysis of CD11b immunoreactivity

Images were digitized in grayscale and CD11b immunostaining was evaluated with the analysis program Scion Image (Scion Corp., USA). A threshold, whose value was set above the mean value ±SEM of the background, was applied for background correction. Inside each frame, the area occupied by gray values above the threshold was automatically calculated. For each level of SNc or CPu, the obtained value was first normalized with respect to vehicle, and values from different levels were averaged thereafter.

Statistics

All data were statistically analyzed with a 1-way ANOVA, followed by Tukey's post hoc test. Results were considered significant at $P < 0.05$.

RESULTS

ST1535 prevented neurodegeneration induced by MPTP in the SNc and CPu

MPTP treatment (20 mg/kg × 4, i.p.) induced a significant loss of TH-positive neurons in the SNc (32%), as measured by stereological counting (Fig. 1). Treatment with ST1535 (2 mg/kg, i.p.) + MPTP completely prevented any loss of TH-positive cells ($n = 4$–5; $F_{(2,11)} = 17.82$; $P < 0.001$).

In addition, total number of cells in the SNc was extrapolated as described in Material and Methods (vehicle: 20,498 ± 1437; MPTP: 13,344 ± 582; MPTP + ST1535: 17,375 ± 612; $n = 4$–5; $F_{(2,11)} = 16.34$; $P < 0.001$).

To determine the effective neurodegeneration, adjacent SNc sections were stained with cresyl violet.

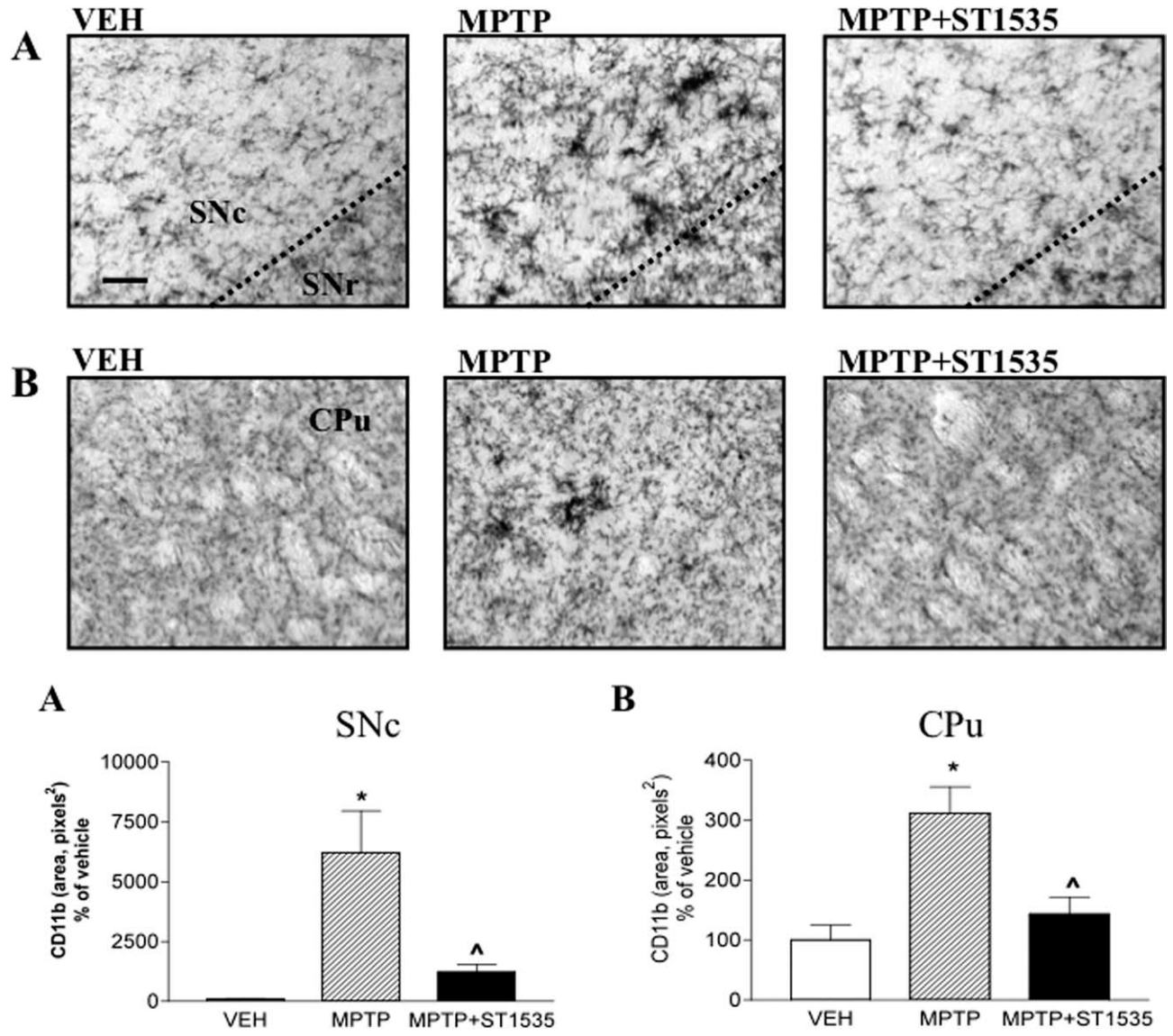

Fig. 3. Immunoreactivity for CD11b in SNc and CPu. Representative sections of SNc (A) and CPu (B) immunostained for CD11b. Mice were treated with MPTP (20 mg/kg once a day for four days, i.p.) plus vehicle or ST1535 (2 mg/kg twice a day during MPTP treatment, once a day the day before and two days after MPTP) and sacrificed three days after MPTP treatment. The area occupied by gray values above a threshold was calculated and expressed as square pixels and as a percentage of staining in vehicle-treated mice in the SNc (A) and CPu (B). Values are expressed as mean ± SEM. $^{*}P < 0.001$ as compared wih the vehicle-treated mice; $^{\wedge}P < 0.001$ as compared with the MPTP-treated mice, by Tukey's post hoc test. $n = 13$ in the SNc and $n = 13$–16 in the CPu. Scale bar, 50 μm.

Stained cells in the SNc confirmed neuron loss with MPTP and the complete prevention of neuron loss with ST1535 (Fig. 1) ($n = 4$–5; $F_{(2,11)} = 43.58$; $P < 0.001$).

MPTP treatment (20 mg/kg × 4, i.p.) induced a similar significant decrease of TH in the CPu (25%), (Fig. 2). Treatment with ST1535 + MPTP completely prevented any loss of TH in the CPu ($n = 5$–6; $F_{(2,13)} = 38.00$; $P < 0.001$).

ST1535 counteracted glial activation induced by MPTP in the SNc and CPu

In vehicle-treated mice, resting microglia and very low levels of CD11b were detected, both in the SNc and CPu. Subchronic MPTP treatment induced a significant increase in CD11b immunoreactivity, and microglia showed an ameboid aspect, in both the SNc and CPu (Fig. 3). Combined treatment with ST1535 (2 mg/kg, i.p.) + MPTP (20 mg/kg × 4, i.p.) completely prevented the increase in CD11b immunostaining in both the SNc and CPu (Fig. 3) ($n = 13$; $F_{(2,36)} = 8.84$; $P < 0.001$ in the SNc and $n = 13$–16; $F_{(2,41)} = 10.63$; $P < 0.001$ in the CPu).

Few astroglial cells were observed in vehicle-treated mice. Moreover, GFAP-positive cells displayed a highly branched morphology with tiny processes and a small body. MPTP treatment (20 mg/kg × 4,

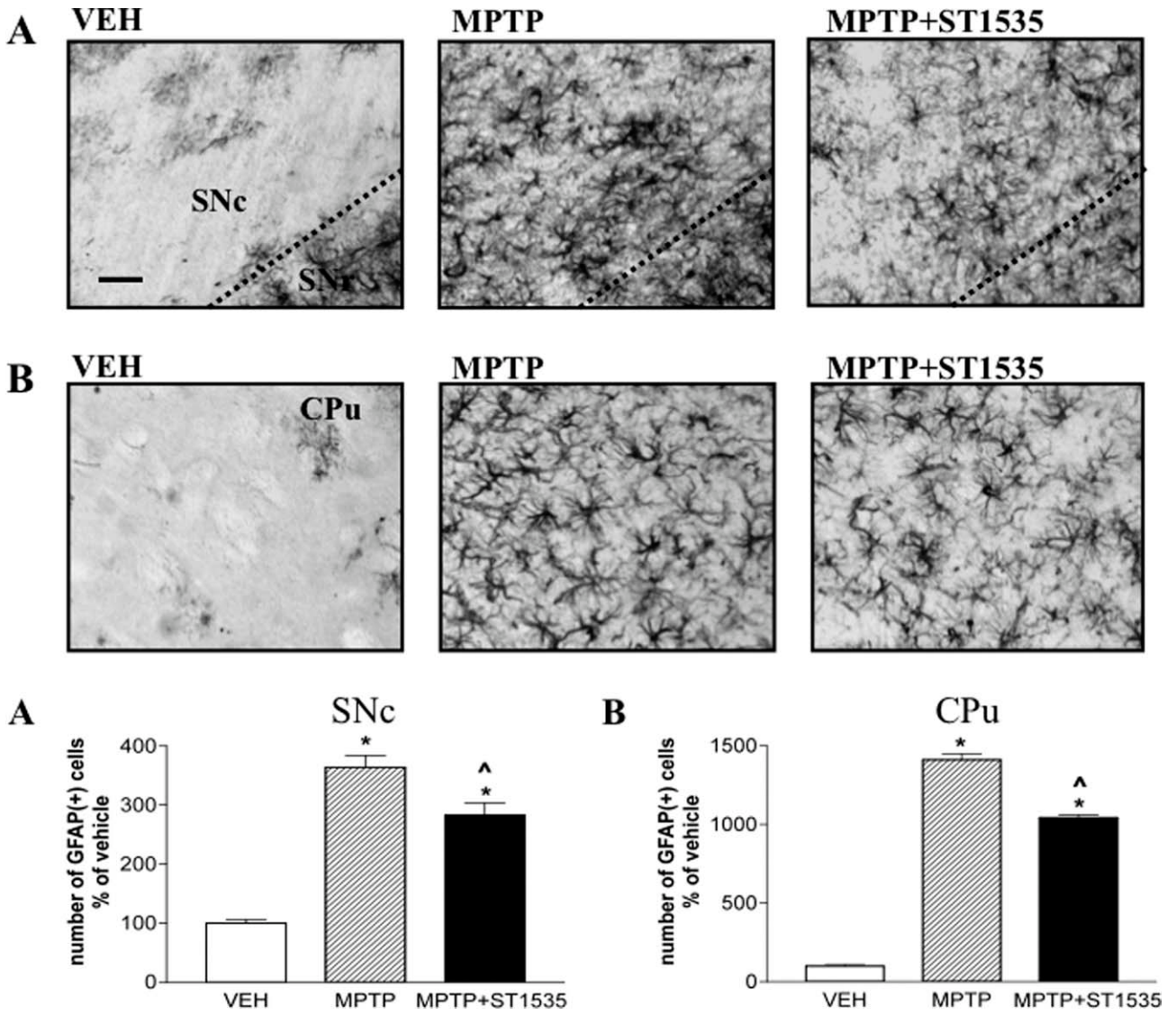

Fig. 4. Immunoreactivity for GFAP in SNc and CPu. Representative sections of SNc (A) and CPu (B) immunostained for GFAP. Mice were treated with MPTP (20 mg/kg once a day for four days, i.p.) plus vehicle or ST1535 (2 mg/kg twice a day during MPTP treatment, once a day the day before and two days after MPTP) and sacrificed three days after MPTP treatment. Graphs show the number of GFAP-positive cells expressed as a percentage with respect to vehicle-treated mice in the SNc (A) and CPu (B). Values are expressed as mean ± SEM. *$P < 0.001$ as compared to the vehicle-treated mice; ^$P < 0.001$ as compared to the MPTP-treated mice, by Tukey's post hoc test. n = 14–15. Scale bar, 50 μm.

i.p.) was associated with a higher number of GFAP-positive cells and cells became hypertrophic (Fig. 4). ST1535 (2 mg/kg, i.p.) significantly but only partially counteracted the MPTP-induced astrogliosis, both in the SNc and CPu (Fig. 4) (n = 14–15; $F_{(2,40)} = 57.94$; $P < 0.001$ in the SNc and n = 14–15; $F_{(2,41)} = 712.47$; $P < 0.001$ in the CPu).

DISCUSSION

Results obtained in the present study show how the A_{2A} receptor antagonist ST1535 prevented MPTP-induced TH-positive cell loss in the SNc. Cresyl violet staining confirmed cell death after MPTP treatment, whereas the combination of MPTP + ST1535 offered complete protection against neurodegeneration. These results are in line with previous evidence that A_{2A} receptor antagonist administration is neuroprotective against the degeneration of dopaminergic neurons induced by MPTP or 6-OHDA in animal models of PD (Chen et al., 2001; Ikeda et al., 2002; Pierri et al., 2005; Wardas, 2002). It should be emphasized that in the present study, as in the majority of neuroprotective studies, the A_{2A} antagonist was given before MPTP administration. Therefore, extrapolation of results to clinical application should take into account

that when diagnosis of PD is made dopaminergic neuron degeneration is already up to 70–80%, and therefore a neuroprotective strategy in the clinic should stop rather than preventing neurodegeneration. This consideration, however, does not affect the relevance of the neuroprotective action of ST1535, considering the different progression of dopaminergic neuron degeneration: rapid in rodent models, very slow (about 5–10 years) in humans.

The mechanism by which A_{2A} receptor blockade mediates neuroprotection in PD remains unclear. A_{2A} receptor stimulation increases glutamate release in the CPu through modulation of neuronal terminals and, possibly, indirectly in subthalamic neurons projecting to the SNc, which are controlled by GABA release from the globus pallidus (Marchi et al., 2002; Marcoli et al., 2003; Melani et al., 2003; Popoli et al., 1995; Schwarzschild et al., 2003). Therefore, a possible glutamate-related mechanism could be envisioned whereby A_{2A} receptor blockade may protect dopaminergic neurons in models of PD by antagonizing the increase in glutamate to toxic levels.

Besides an active role of glutamate in neurodegeneration, several findings have suggested that neuroinflammation may play a role in the pathogenesis of neurodegeneration in PD, since reactive astrogliosis and microgliosis have been described in the SNc of PD patients and MPTP-treated primates (Barcia et al., 2004; Hunot and Hirsch, 2003). In addition, activation of microglia has been shown to be a primary factor in the neurodegenerative process of PD (Meredith et al., 2005), and blockade of microglial reactivity in mice protects dopaminergic neurons from acute MPTP toxicity (Teismann and Ferger, 2001; Wu et al., 2002).

Recent data have shown that A_{2A} receptor antagonism counteracts neuroinflammatory processes (Carta et al., 2009; Huang et al., 2006) by inhibiting astroglial and microglial activation (Ikeda et al., 2002; Pierri et al., 2005). In accordance with this, A_{2A} receptor knockout mice displayed reduced astrogliosis and microgliosis after subchronic MPTP administration (Carta et al., 2009).

The results obtained in the present study show complete inhibition of microglial reactivity after ST1535 treatment, in line with previous evidence obtained with other A_{2A} receptor antagonists on neuroinflammation induced by MPTP administration in mice (Carta et al., 2009; Ikeda et al., 2002; Pierri et al., 2005).

MPTP treatment also induced intense astrogliosis in both the SNc and CPu, a phenomenon characterized by increased proliferation, hypertrophy, and elongation of cellular GFAP-positive processes (Neary et al., 1996; Ridet et al., 1997). Increased GFAP immunoreactivity has also been detected in the SNc of monkeys 1 year after chronic MPTP intoxication and in postmortem CPu from parkinsonian patients (Barcia et al., 2004; Langston et al., 1999; McGeer et al., 1988). ST1535 displays positive effects in counteracting astrogliosis; however, in contrast with microglia, reactive astroglia were only partially inhibited by ST1535 both in the SNc and CPu. The partial antagonism of astroglia activation is not surprising since astroglia is, in general, less sensitive than microglia to A_{2A} receptor antagonism-mediated anti-inflammatory effects and is in line with previous finding obtained in A_{2A} receptor knockout mice (Carta et al., 2009).

The counteraction of dopaminergic neuron degeneration and glial activation shown by ST1535 in the present study and the improvements observed in motor deficits when the drug is used in addition to low doses of L-DOPA (Tronci et al., 2007) make this a very interesting alternative drug for adjunctive therapy in PD.

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