SEMMELWEIS UNIVERSITY

JANOS SZENTAGOTHAI NEUROSCIENCE PhD SCHOOL

TIME COURSE OF NEURONAL DAMAGE AND RECOVERY INDUCED BY MDMA: EXPRESSION AND DISTRIBUTION OF SEROTONIN TRANSPORTER IN THE RAT BRAIN

PhD thesis

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TABLE OF CONTENTS

1. ABBREVIATIONS

4V	fourth ventricle
5-HIAA	5-hydroxyindole acetic acid
5-HIAL	5-hydroxyindole acetaldehyde
5-HT	5-hydroxytryptamine, serotonin
5-HTOL	5-hydroxytryptophol
5-HTT	serotonin transporter
AANAT	serotonin N-acetyltransferase
Ach	acethylcholine
ANOVA	analysis of variance
AW	active wake
Aq	aqueduct
BMR	basal metabolic rate
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CSF	cerebrospinal fluid
CYP	cytochrome P450
DA	dopamine
DAB	3,3'-diaminobenzidine
DAG	diacyl-glycerol
DHMA	3,4-dihydroxymethamphetamine (*N*-methyl- α-methyldopamine)
DHT	dihydroxytryptamine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide- triphosphate
DR	dorsal raphe nucleus
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
EMG	electromyogram
GABA	gamma-aminobutyric acid

GD	dentate gyrus
HHA	3,4-dihydroxyamphetamine
HMA	4-hydroxy-3-methoxyamphetamine
HMMA	4-hydroxy-3-methoxy-methamphetamine
HPLC-ED	high-performance liquid chromatography with electrochemical detection
IP_3	inositol-triphosphate
IR	immunoreactive
LH	lateral hypothalamic area
L-DOPA	3,4-dihydroxy-L-phenylalanine
L-NAME	N-nitro-L-arginine methylester
MAO	monoamine- oxidase
MDA	3,4-methylenedioxyamphetamine
MDAE	3,4-methylenedioxyethamphetamine
MDMA	3,4-methylendioxymethamphetamine
MFB	medial forebrain bundle
MHPG	3-methoxy-4-hydroxy-phenylglycol
ml	medial lemniscus
mPRF	medial pontine reticular formation
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR	median raphe nucleus
mRNA	messenger ribonucleic acid
MRS	magnetic resonance spectroscopy
NAA	N-acetylaspartate
NE	norepinephrine
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
NOS	nitric oxide synthase
NREM	non- rapid eye movement
PAGvl	periaqueductal grey, ventrolateral part
PAGdm	periaqueductal grey, dorsomedial part
PB	phosphate buffer

PBS	phosphate buffered saline
PCA	parachloroamphetamine
PET	positron emission tomogrpahy
PH	posterior hypothalamic nucleus
PLC	phospholipase C
PPTG	peripeduncular tegmental nucleus
PVN	paraventricular nucleus
PW	passive wake
py	pyramidal tract
REM	rapid eye movement
RMg	raphe magnus nucleus
RNAse	ribonuclease
ROb	raphe obscurus nucleus
RPa	raphe pallidus nucleus
SAL	saline
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SMC	somatomotor cortex
SNRI	serotonin- norepinephrine reuptake inhibitor
SPECT	single photon emission computed tomography
SSC	saline- sodium citrate
SSCX	somatosensory cortex
SSRI	selective serotonin reuptake inhibitor
SWS	slow wave sleep
TBST	Tris-Buffered Saline Tween-20
TMN	tuberomamillary nucleus
TPH	tryptophan- hydroxylase
VLPO	posteroventral preoptic areas
VMAT	vesicular monoamine transporter
xscp	decussation of superior cerebellar peduncles

2. INTRODUCTION

2.1. MDMA pharmacology and history

The ring-substituted amphetamine derivative (±) 3,4-methylendioxymethamphetamine (MDMA; *Ecstasy*) has become a widely abused psychoactive drug among young people in the United States, Europe and elsewhere. Ecstasy has become second in popularity to cannabis as an illicit recreational drug (Pope et al., 2001). The original street names of MDMA are "Empathy", Adam, E, XTC, and just plain X (McDowell and Kleber, 1994; Parrott, 2004). MDMA is a phenylethylamine, structurally similar to amphetamine (**Figure 1**, Green et al., 1995; Green et al., 2003; McKenna and Peroutka, 1990).

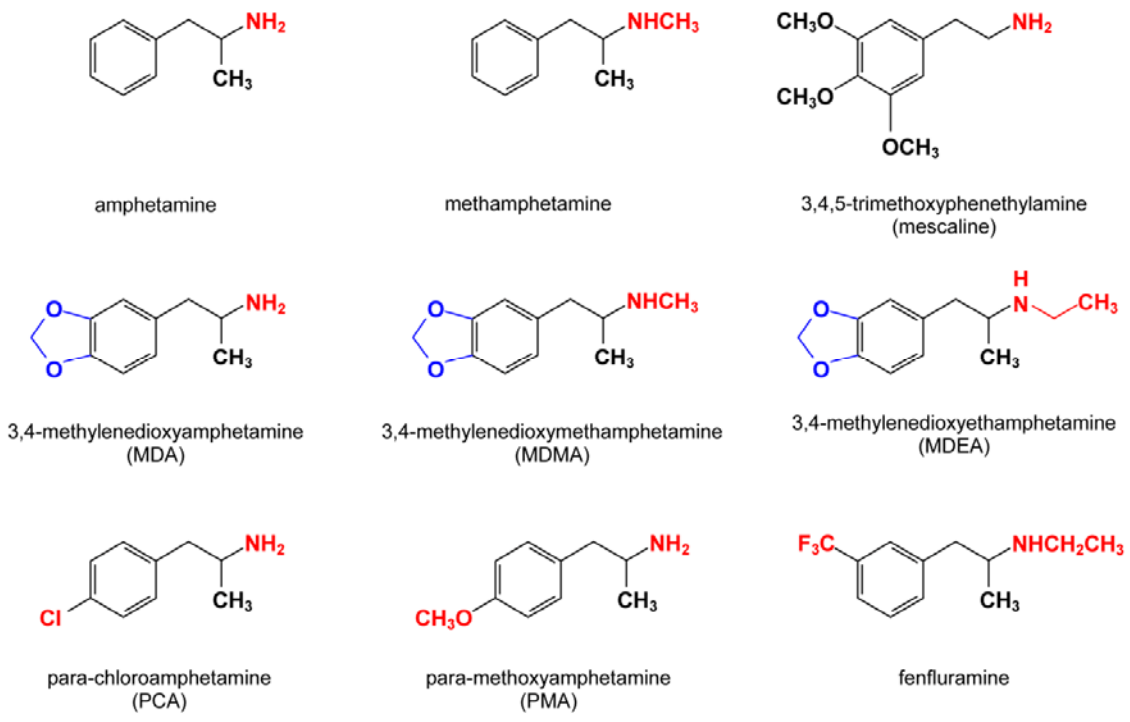


FIGURE 1. Chemical structures of MDMA and related compounds

MDMA was first synthesized around 1912, by Merck in Darmstadt, Germany (Liester et al., 1992; McDowell and Kleber, 1994). MDMA has often been said to have been originally patented for use as an appetite suppressant but it was never produced

commercially nor did it achieve clinical use for this indication. In the original patent specification there were no indications for plans to develop an anorectic drug (Freudenmann et al., 2006). Probably it was a precursor agent in a new chemical pathway which was patented in order to avoid an existing patent for the synthesis of the clotting agent hydrastatine. MDMA was mentioned only casually and without a name as one of many chemical intermediates, and appeared only as a chemical formula (methylsafrylamin). MDMA was neither studied in animals nor humans at Merck around 1912. The first basic pharmacological tests using MDMA were performed by the company's chemists decades later (1927, 1952) (Bernschneider-Reif et al., 2006). The United States Army experimented with the compound during the 1950s (Hardman et al., 1973). MDMA was first used by humans in the late 1960s. MDMA remained on the laboratory shelves until it was re-synthesised in the early 1970's by Alexander Shulgin, a research chemist employed to develop structural variants of mescaline and several synthetic amphetamine derivatives (Shulgin, 1986).

The ability of MDMA to enhance feelings of openness and trust in its users was the basis for the use of the drug as an adjunct to psychotherapy during the 1980s (Downing, 1986; Greer and Tolbert, 1986; Grinspoon and Bakalar, 1986; Naranjo et al., 1967).

MDMA has been illegal since it was classified as a Schedule I drug by the United States Drug Enforcement Administration in July 1985 (Liester et al., 1992), due to its high abuse potential, lack of clinical\ medical application, lack of accepted safety for use under medical supervision and evidence that MDA (3,4-methylenedioxyamphetamine), a related compound and major MDMA metabolite, induced serotonergic nerve terminal degeneration in the rat brain (Ricaurte et al., 1985; Schmidt et al., 1986; Stone et al., 1986).

In spite of its illegal status, since the mid 1980s it has become popular as a recreational drug, often being taken at "rave" or "techno" parties, particularly in large dancing clubs (Green et al., 1995).

Ecstasy comes in a variety of colours, shapes, and pill sizes, decorated with a wide variety of designs or logos. As with any illicitly prepared and obtained recreational drug, both doses and purity vary greatly (Baggott et al., 2000; Renfroe, 1986), but tablets have regularly been found to contain between 80 and 150 mg of MDMA. Most

Ecstasy pills contain MDMA but studies also found several other constituents e.g. MDA, MDAE (3,4-methylenedioxyethamphetamine), ketamine, amphetamine, codeine, caffeine, ephedrine, salicylates or placebo (Baggott et al., 2000; Parrott, 2001; Schifano et al., 1998).

Globally, after cannabis, amphetamines and ecstasy are among the most commonly consumed illicit drugs. Results of a 2003 general Hungarian population survey reveal that lifetime prevalence is 3.1% for ecstasy use (European Monitoring Centre for Drugs and Drug Addiction, 2008). The 2006 survey showed that 20.3% of 15–17-year-old students have used an illicit substance at least once. Lifetime prevalence of ecstasy use was 6.1%. The use of all drugs was found to be influenced by gender: prevalence of drug use among male students was higher than among female students. Available data for the 18–34-year age group showed that 20.1% reported lifetime experience with illegal drugs. Lifetime experience with ecstasy was in second place, at 5.6% (European Monitoring Centre for Drugs and Drug Addiction, 2008).

2.2. Serotonergic system

2.2.1. Serotonin

Serotonin, 5-hydroxytryptamine (5-HT) is a biogenic monoamine that is widely distributed in the body (**Figure 2**). Serotonin was first isolated in the 1950s from the blood as a serum factor that increased smooth muscle tone (Amin et al., 1954; Twarog and Page, 1953). 5-HT is present in significant concentrations in the central nervous system (CNS) and in the periphery.

Serotonin in the CNS acts as a neurotransmitter and modulates a variety of functions (Hoyer et al., 2002; Zifa and Fillion, 1992) such as mood (Meltzer, 1989; Meltzer, 1990), anxiety (Nutt et al., 1990), aggression, impulsiveness (Berman et al., 1997; Morgan, 1998), pain (Richardson, 1990), cognition, memory (Altman and Normile, 1988), sleep (Wauquier and Dugovic, 1990), appetite (Curzon, 1990), emesis (Bunce et al., 1991), motor activity (Jacobs and Fornal, 1997), body temperature (Schwartz et al., 1995), epilepsy (Bagdy et al., 2007), endocrine function (Jorgensen,

2007), circadian rhythms (Wesemann and Weiner, 1990), social and reproductive behaviour (Charnay et al., 1996; Gorzalka et al., 1990).

An important pool of 5-HT outside the CNS can be found in the enterochromaffin cells of the gut and in blood platelets (Mitro et al., 2006). The greatest concentration of 5-HT (80- 90%) is found in the enterochromaffin cells. 5-HT participates in numerous physiological and homeostatic processes, such as gastrointestinal peristalsis, blood coagulation and the maintenance of blood pressure (Awabdy et al., 2003; Cote et al., 2004). The peripherally synthetized serotonin does not effect activity of the CNS, because is not able to cross blood-brain barrier.

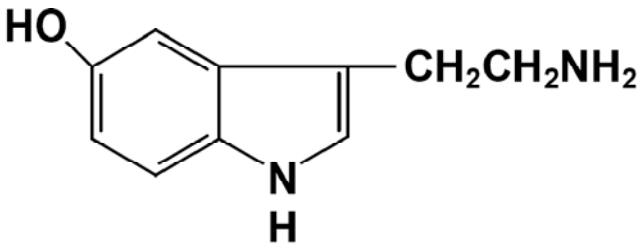


FIGURE 2. The chemical structure of serotonin

2.2.2. Serotonin synthesis in the central nervous system

Serotonin is synthesized from the amino acid precursor tryptophan through a two-step process involving the ***tryptophan-hydroxylase*** (TPH) and then a decarboxylation catalyzed by the ***L-aromatic acid decarboxylase*** (**Figure 4**, Tohyama and Takatsuji, 1998). The TPH- mediated reaction is the rate-limiting step in the pathway. Serotonin is packaged into synaptic vesicles and released into the synapse in response to depolarizing stimuli (**Figure 3**). Once in the synapse, serotonin can interact with both pre- and postsynaptic receptors. However, immediately after reacting with pre- and postsynaptic receptors, it is critically important that serotonin be removed from the synaptic cleft. Presynaptic plasma membrane transporters control synaptic actions of 5-HT by rapidly clearing the released 5-HT (Barker and Blakely, 1995; Jayanthi and Ramamoorthy, 2005; Lesch, 1997). Once 5-HT is in the serotonergic neuron, it can be enzimatically degraded by MAO (monoamine- oxidase) or taken back into the storage

vesicles via vesicular monoamine transporter (VMAT) for reuse (**Figure 3**, Lesch, 1997; Liu and Edwards, 1997).

Metabolism of 5-HT mainly occurs in the intestine, kidney, liver and lung by the action of MAO to form the intermediate 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate is either oxidized by aldehyde dehydrogenase to 5-hydroxyindoleacetic acid (5-HIAA), or reduced by alcohol dehydrogenase or alcohol reductase to 5-hydroxytryptophol (5-HTOL) (Beck and Helander, 2003; Musshoff, 2002).

Melatonin is biosynthesized from serotonin in the pineal gland, although there are some additional pineal sites such as the retina and gastrointestinal tract. The rate-limiting step in the synthesis of melatonin from serotonin is the one catalyzed by the cytoplasmic enzyme serotonin N-acetyltransferase (AANAT) because its level and activity also shows daily rhythm, with nighttime levels being higher than daytime values. In contrast, the other enzyme, hydroxyindole-O-methyltransferase displays no such day-night changes. Therefore, it is the AANAT rhythm that underlies the rhythmic synthesis of melatonin, and AANAT has been called the melatonin rhythm enzyme (Zheng and Cole, 2002).

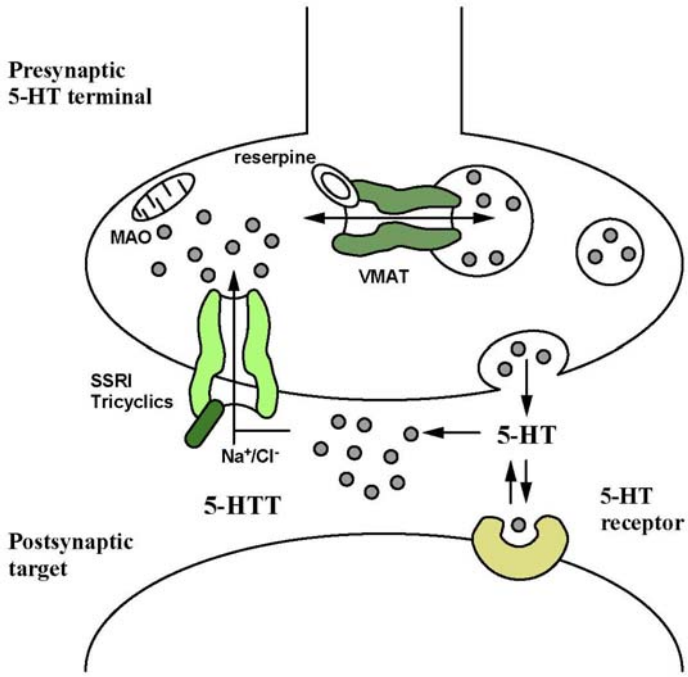


FIGURE 3. Serotonergic synapse (from Lesch et al. 1997)

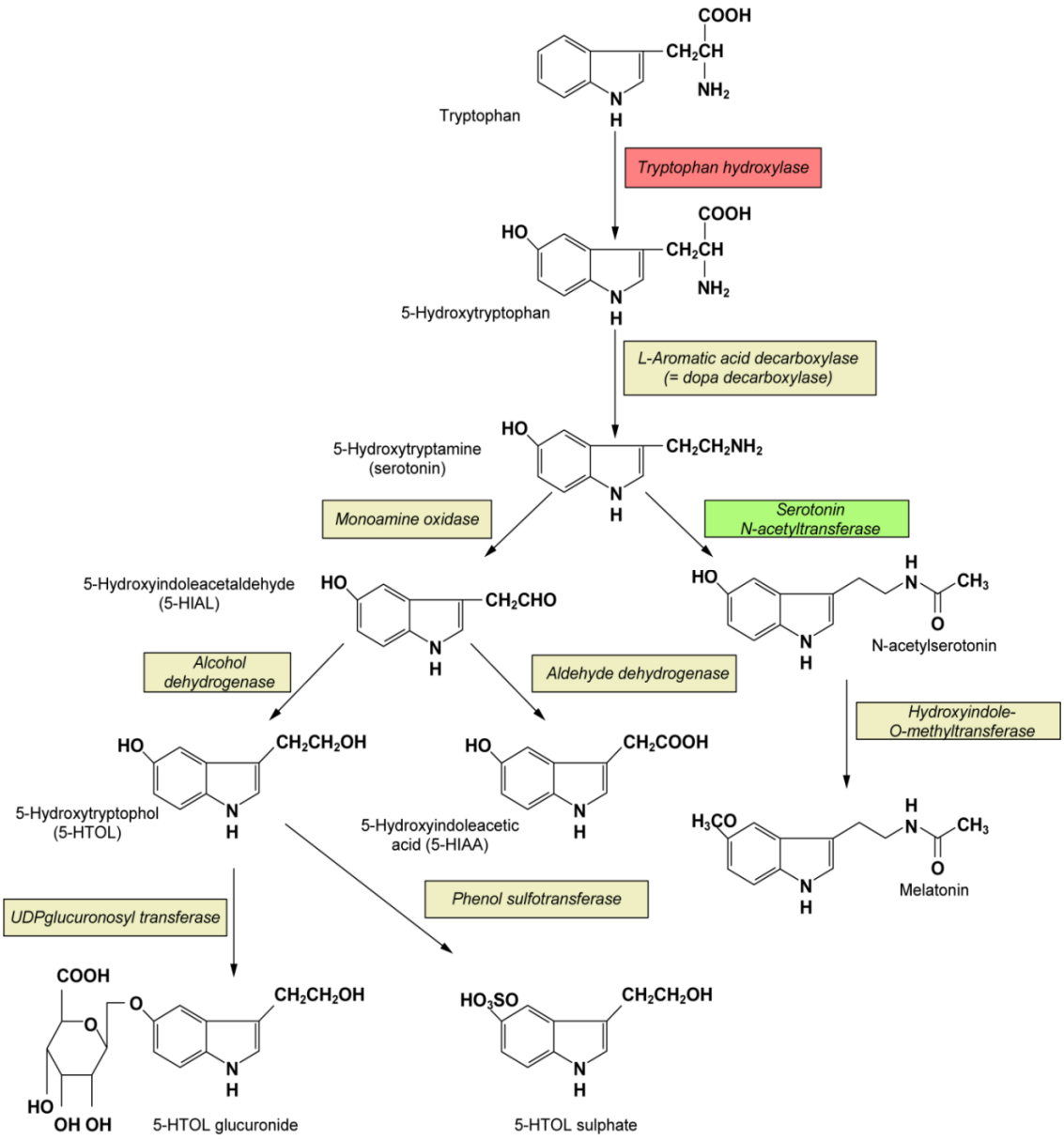


FIGURE 4. Synthesis and metabolism of serotonin

(the rate-limiting step of serotonin synthesis is highlighted in red colour and the rate-limiting step of melatonin synthesis is highlighted in green colour)

Abbreviations: 5-HIAL - 5-hydroxyindole acetaldehyde; 5-HIAA - 5-hydroxyindoleacetic acid, 5-HTOL - 5-hydroxytryptophol

2.2.3. Distribution of serotonergic cells

The serotonin-producing neurons in the brainstem raphe nuclei have the largest and most complex efferent system in the brain. The special nature of the raphe brainstem neurons was recognized by the classical neuroanatomist Ramón y Cajal, who described these giant neurons in the brainstem midline but was unable to follow their projections (Ramón y Cajal, 1911). Dahlström and Fuxe's work with histochemical fluorescence provided details concerning the anatomical architecture of brain 5-HT (Dahlström and Fuxe, 1964). Serotonergic neurons were first demonstrated by the histofluorescence method (Falck et al., 1962). The original histofluorescence studies were limited by the weak and rapidly falling fluorescence of 5-HT. The low sensitivity of histofluorescence did not permit detection of fine axons in the forebrain, so the density of innervation was initially underestimated. It was not possible to visualize the distribution of 5-HT containing neurons until the advent of immunohistochemistry using 5-HT (Steinbusch, 1981), its precursor, 5-hydroxytryptophan (Touret et al., 1987), and its synthetizing enzyme, tryptophan hydroxylase (Weissmann et al., 1987) antibodies.

5-HT neurons develop in the early fetal life as two separate clusters within the brainstem: one rostral and one caudal group (Aitken and Törk, 1988). The rostral group gives rise to almost all ascending 5-HT fibres, whereas the caudal group gives rise to the majority of descending fibres (Aitken and Törk, 1988; Halliday et al., 1995; Wallace and Lauder, 1983).

The cells which produce 5-HT are found only in restricted areas of the brain, namely in the raphe nuclei groups which exist from the midbrain to the medulla oblongata (Dahlström and Fuxe, 1964; Taber et al., 1960). These areas are classified into nine regions (B1-B9) (Dahlström and Fuxe, 1964). The B1, B2, B3 and B4 groups develop from 5-HT cells born in the caudal group, whereas the B5, B6, B7, B8 and B9 groups develop from 5-HT cells born in the rostral group (Törk, 1990). Serotonergic axons that innervate the forebrain arise from neurons within the mesencephalic raphe nuclei.

B1 nucleus raphe pallidus (RPa)\ caudal ventrolateral medulla

These are the most ventrally placed 5-HT neurons in the medulla oblongata (**Figure 5, 6**). The majority of neurons are located between the pyramids in RPa, although occasional 5-HT neurons are found within the pyramids and on the ventral surface of pyramids (Toth et al., 2006). 5-HT neurons are found from the level of the caudal pole of the facial nucleus to the level of the pyramidal decussation. The most rostral 5-HT neurons in RPa are contiguous with 5-HT neurons in the raphe magnus nucleus (Azmitia and Whitaker-Azmitia, 2000; Halliday et al., 1995; Takeuchi, 1988).

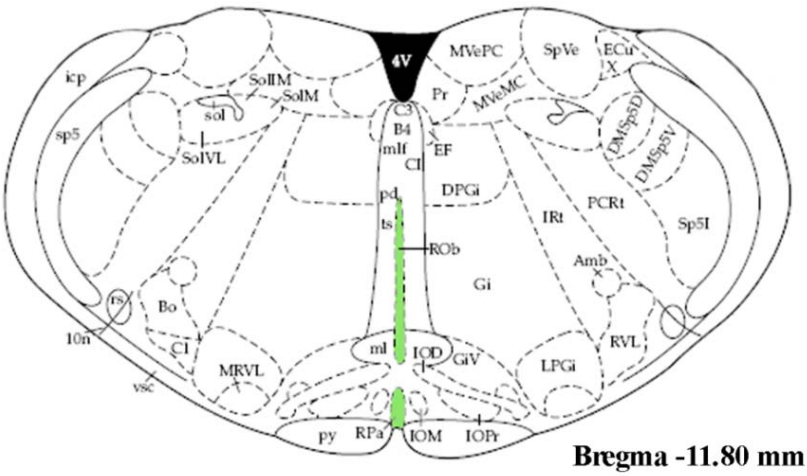


FIGURE 5. The location of serotonergic cell bodies in the rat brain in the nucleus raphe pallidus and obscurus (the nucleus is highlighted in green colour)
Abbreviations: RPa - nucleus raphe pallidus; ROb - nucleus raphe obscurus; py – pyramidal tract; ml – medial lemniscus; 4V – fourth ventricle
(from Paxinos and Watson 2005)

B2 nucleus raphe obscurus\ B4 central grey matter of the medulla oblongata

5-HT neurons in the nucleus raphe obscurus (ROb) are found along the midline throughout the medulla oblongata as two vertical parallel sheets of neurons dorsal to the inferior olive and pyramids (**Figure 5**). The majority of neurons are located in the ventral half of the medulla oblongata, although occasional 5-HT neurons are located within the central gray structures. 5-HT neurons are found from the level of the rostral inferior olive to the level of the pyramidal decussation. The most rostral 5-HT neurons in the ROb are contiguous with 5-HT neurons in the raphe magnus nucleus (Azmitia and Whitaker-Azmitia, 2000; Halliday et al., 1995; Takeuchi, 1988).

B3 nucleus raphe magnus (RMg)\ rostral ventrolateral medulla

The RMg is located in the rostral ventromedial medulla of the brainstem. The majority of neurons are found dorsal to the pyramids in the ventral half of the tegmentum of the rostral medulla oblongata and caudal pons (**Figure 6**). The distribution of B3 neurons resembles a triangle in transverse section. The most caudal 5-HT neurons in RMg are contiguous with the midline 5-HT neurons in RPa and ROb (Azmitia and Whitaker-Azmitia, 2000; Halliday et al., 1995; Takeuchi, 1988). Axons of the RMg project to the spinal cord, terminating primarily in the dorsal horn (Jones and Light, 1990).

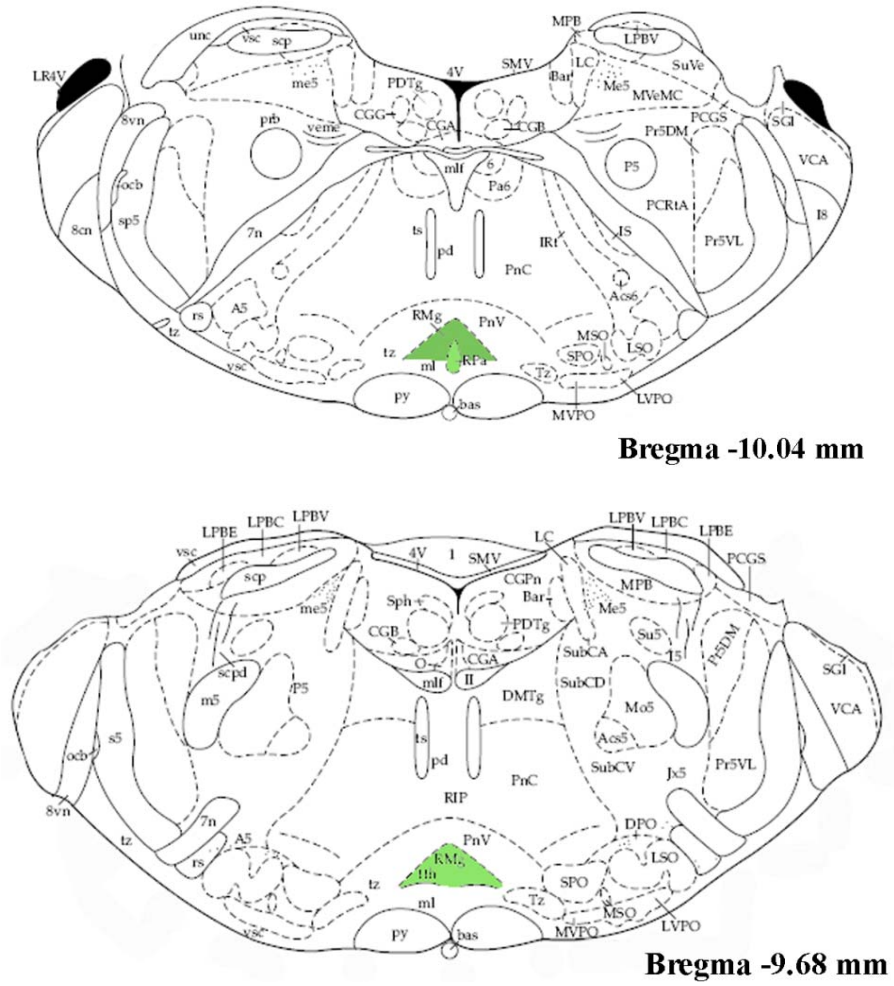


FIGURE 6. The location of serotonergic cell bodies in the rat brain in the nucleus raphe magnus (the nucleus is highlighted in green colour)

Abbreviations: RMg – nucleus raphe magnus; RPa – nucleus raphe pallidus; py – pyramidal tract; ml – medial lemniscus; 4V – fourth ventricle

(from Paxinos and Watson 2005)

B5 pontine median raphe nucleus\ B8 midbrain median raphe nucleus\ caudal linear nucleus

5-HT neurons in the B5 group are located in the pontine raphe nucleus. The median raphe nucleus (MR, central superior, B8) lies within the central portion of the reticular formation in the midbrain tegmentum (**Figure 7**). The larger B8 group of 5-HT neurons is found throughout the midline raphe of the rostral pons and caudal midbrain.

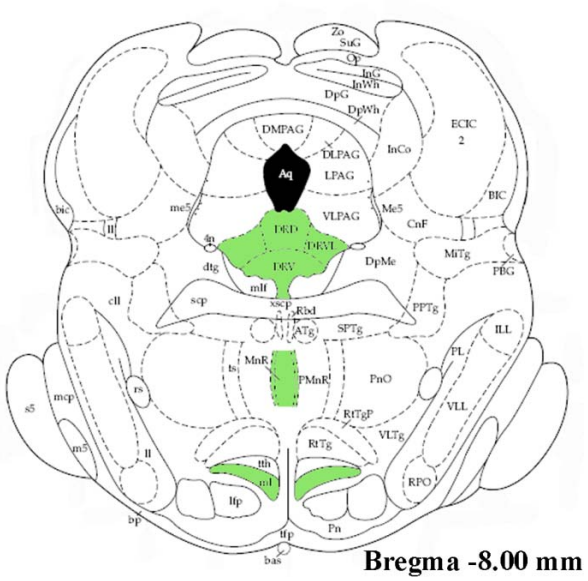
Dorsal 5-HT neurons are contiguous with 5-HT cells in the (DR) raphe nucleus, whereas the ventrally placed 5-HT neurons are contiguous with the B9 group. MR fibres descend along the midline within the brainstem and mainly ascend within the MFB (medial forebrain bundle) in the forebrain (Azmitia and Whitaker-Azmitia, 2000; Halliday et al., 1995; Palkovits et al., 1977; Takeuchi, 1988).

B6 pontine dorsal raphe nucleus\ B7 midbrain dorsal raphe nucleus

The largest and most dense group of serotonergic neurons is the dorsal raphe nucleus (DR, B7) which lies in the periaqueductal grey matter (**Figure 7**). It contains 235.000 5-HT immunoreactive (IR) neurons (Baker et al., 1990) in the human brain. This nucleus extends from the levels just caudal to the oculomotor nucleus down to the rostral portion of the fourth ventricle. The DR is continuous caudally with a smaller group of 5-HT cells (B6) that lies along the midline and the floor of the fourth ventricle (Azmitia and Whitaker-Azmitia, 2000; Halliday et al., 1995; Takeuchi, 1988).

B9 medial lemniscus

5-HT neurons in the B9 group are associated with the medial lemniscus (**Figure 7**). The B9 cell group consist of a scattered group of 5-HT neurons that lies along the dorsal surface of the medial lemniscus in the ventrolateral tegmentum (Azmitia and Whitaker-Azmitia, 2000; Halliday et al., 1995; Takeuchi, 1988).



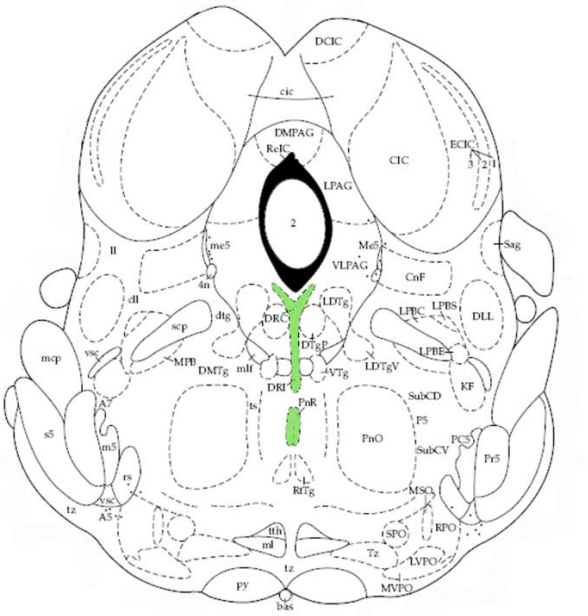


FIGURE 7. The location of serotonergic cell bodies in the rat brain in the dorsal and median raphe nucleus, and medial lemniscus (the nucleus is highlighted in green colour) *Abbreviations: DRD - dorsal raphe nucleus; dorsal part, DRV - dorsal raphe nucleus, ventral part; DRVL – dorsal raphe nucleus, ventrolateral part; DRC – dorsal raphe nucleus, caudal part; DRI – dorsal raphe nucleus, interfascicular part; MnR – median raphe nucleus; ml – medial lemniscus; xscp – decussation of superior cerebellar peduncles; PPTG –peripeduncular tegmental nucleus; PnO – pontine reticular nucleus, oral part; PnR- pontine raphe nucleus* (from Paxinos and Watson 2005)

2.2.4. Main 5-HT pathways

Serotonergic axon terminals have been found in widespread areas of the forebrain and throughout the brainstem and spinal cord (Brownstein and Palkovits, 1984; Palkovits et al., 1977; Steinbusch, 1981; Törk, 1990). The extent to which neurons of each raphe nucleus project to various forebrain nuclei has been studied with a variety of techniques, including selective lesions (Conrad and Pfaff, 1976; Meyer-Bernstein and Morin, 1996; Palkovits et al., 1977) and both retrograde or anterograde label transport (Datiche et al., 1995; De Olmos and Heimer, 1980; Kohler et al., 1982; Li et al., 1993).

A series of studies employing small intracerebral lesions (Anden et al., 1971; Ungerstedt, 1971) indicated that most 5-HT nerve terminals in the forebrain arise from raphe nuclei in the midbrain and that the axons ascend through the lateral hypothalamus within the medial forebrain bundle (Azmitia and Segal, 1978; Conrad et al., 1974; Moore and Heller, 1967). Some 5-HT cells lie outside the raphe nuclei (e.g. solitary nuclear complex and dorsomedial hypothalamus) (Brownstein and Palkovits, 1984; Hoffman et al., 1998).

Anatomical evidence indicates that, in contrast to classical 5-HT neurotoxins, MDMA destroys only axon terminals whilst cell bodies are left apparently intact (Molliver et al., 1990; Molliver et al., 1989; Wilson et al., 1989). Furthermore, there is evidence that MDMA causes a selective loss of 5-HT axons of a particular morphological subtype, the so called “fine” 5-HT fibres, whilst sparing “beaded” 5-HT fibres (Molliver et al., 1989; O'Hearn et al., 1988). It has also been proposed that MDMA is more toxic to 5-HT neurons projecting from the DR than to those from the MR. 5-HT axon terminals arising from the MR nucleus have large, spherical varicosities (typically 3 μm in diameter), giving these axons a characteristic beaded appearance (**Figure 8**). In contrast, axons that arise from the DR nucleus are of very fine caliber and typically have minute, pleomorphic varicosities that are often granular or fusiform in shape.

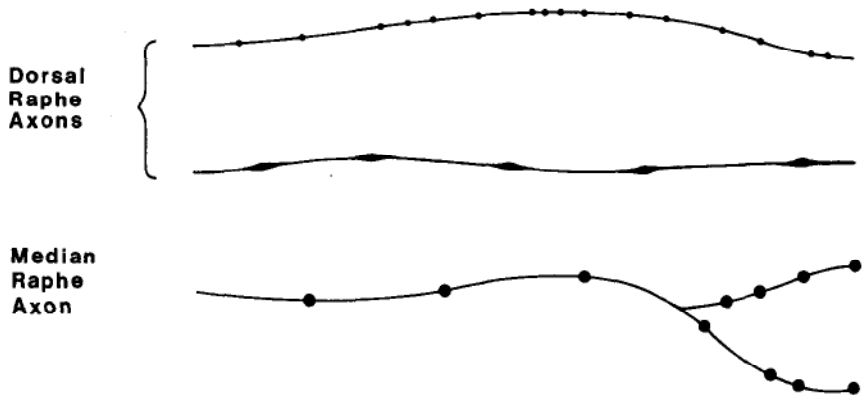


FIGURE 8. A schematic representation showing the two classes of axons (from Molliver et al. 1989)

The first axon transport studies suggested that the DR nucleus projects preferentially upon the cerebral cortex and the striatum while the MR innervates primarily the hippocampus and the hypothalamus. Several studies applying more sensitive methodology demonstrated that the projections were far more complex and that there is considerable overlap in raphe projections to the forebrain. Azmitia and Segal showed that the DR and MR project directly to the forebrain and give multiple, anatomically distinct ascending fibre bundles (Azmitia and Segal, 1978). The terminal distributions of DR and MR ascending projections converge, so that most areas of cerebral cortex are innervated by both nuclei, with regional differences in the relative contribution from each nucleus. Topographical studies which used highly sensitive retrograde transport methods have shown that the cells within different regions of the raphe nuclei project to separate areas of cortex (Kohler and Steinbusch, 1982; O'Hearn and Molliver, 1984; Waterhouse et al., 1986).

2.2.5. Serotonin transporter (5-HTT)

The serotonin transporter (**Figure 9**) is similar to other biogenic amine transporters (e.g. norepinephrine (NE) and dopamine (DA) transporters). 5-HTT is a member of the family of sodium (Na+) and chloride (Cl-) dependent transporters (Amara and Kuhar, 1993; Borowsky and Hoffman, 1995; Brownstein and Hoffman, 1994; Ramamoorthy et al., 1993). The transporter gene has been cloned from rat brain,

human brain, and human placenta (Blakely et al., 1991; Hoffman et al., 1991; Lesch et al., 1993). Molecular weight of 5-HTT is 60-80 kDa. 5-HTT cDNAs are highly conserved among species. In the rat and human species, each 5-HTT cDNA codes approximately 630 amino acids and shares more than 90% common sequences (Gregor et al., 1993; Ramamoorthy et al., 1993). 5-HTT proteins are commonly featured with 12 hydrophobic transmembrane segments, intracellular N- and C-terminals, glycosylated sites in the second external loop, a potential kinase C phosphorilation site in the N- and C-terminals, and a leucine zipper motif in the second transmembrane domain (Lesch, 1997). The N- and C-terminals and the third external loop are the least conserved regions and have been used for discrimination among 5-HTTs. Serotonin transporter, like serotonin, is seen most often in the raphe nuclear complex (Lesch et al., 1993; Rattray et al., 1999). 5-HTTs have also been seen in the amygdala, thalamus, hypothalamus, substantia nigra, and locus coeruleus. In addition, the 5-HTT is expressed in the gut, placenta, lungs, lymphocytes and blood platelets.

Serotonin transporter, the mechanism for 5-HT high affinity uptake, is the essential component for the termination of 5-HT transmission. It plays an important role in the reuptake of 5-HT and subsequently terminates 5-HT transmission. 5-HTT plays a key role in the presynaptic regulation of 5-HT transmission. 5-HTT is itself a target for many drugs such as antidepressants, several neurotoxic agents including the amphetamine derivative MDMA, fenfluramine, a neurotoxic metabolit of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and its analogues (Charnay et al., 1996; Zhou et al., 2000). MDMA is a substrate of the 5-HTT, displacing 5-HT from its storage vesicle, and ultimately inducing 5-HT efflux by a carrier-dependent process. MDMA binds to all three presynaptic monoamine transporters, with highest affinity for the 5-HT transporter. Affinities for the NE and DA transporters are at least 10-fold less (Battaglia et al., 1988; Steele et al., 1987). Binding at both the 5-HT and DA transporters is stereoselective, the S(+)-isomer is more potent, whereas no stereoselectivity is evident at the NE transporter (Steele et al., 1987).

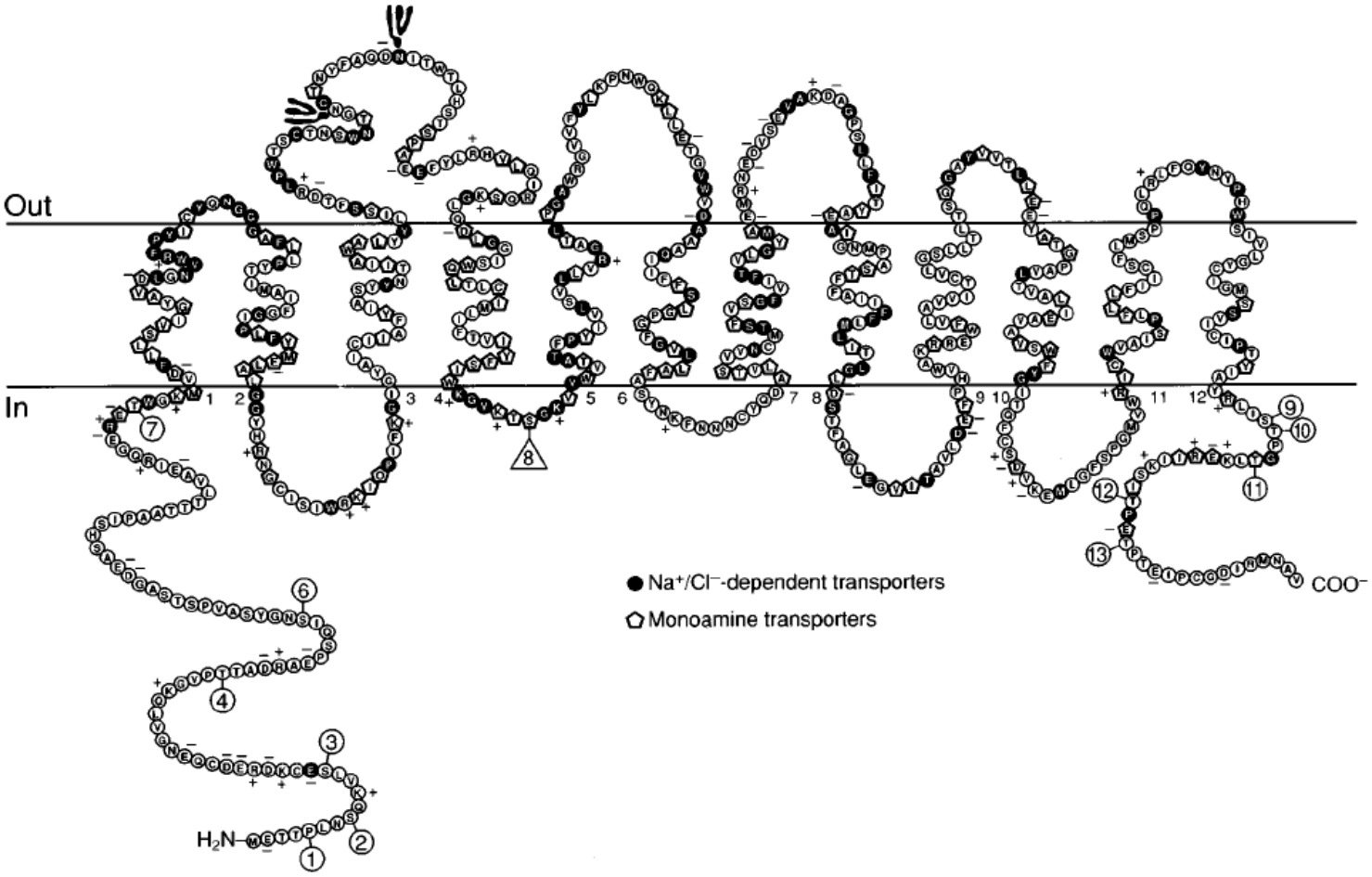


FIGURE 9. The structure of 5-HTT in the rat (from Hoffman et al. 1998)

2.2.6. Serotonin receptors

Serotonin receptors represent one of the most complex families of neurotransmitter receptors and are classified into 7 types (Table 1), 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 and each type has subtypes (Barnes and Sharp, 1999; Hoyer et al., 1994; Hoyer et al., 2002). These receptors are localized in the brain and in the peripheral organs (e.g. gut, cardiovascular system and blood) (Hoyer et al., 2002), but their distribution is not homogeneous. The majority of 5-HT receptors are postsynaptic but receptors such as 5-HT_{1A} and 5-HT_{1B} are mainly presynaptic and modulate serotonin release. Except for the 5-HT_3 receptor, which is a ligand-gated cation channel, other 5-HT receptors belong to the G-protein coupled seven transmembrane receptor superfamily (Aghajanian and Sanders-Bush, 2002; Barnes and Sharp, 1999; Pauwels, 2000)

TABLE 1. Classification of serotonin receptors
Abbreviations: cAMP - cyclic adenosine monophosphate; IP_3 - inositol-triphosphate; DAG - diacyl-glycerol; PLC - phospholipase C

	5-HT$_1$	***5-HT$_2$***	***5-HT$_3$***	***5-HT$_4$***	***5-HT$_5$***	***5-HT$_6$***	***5-HT$_7$***
Subtypes	5-HT_{1A}	5-HT_{2A}	5-HT_{3A}		5-HT_{5A}		
	5-HT_{1B}	5-HT_{2B}	5-HT_{3B}		5-HT_{5B}		
	5-HT_{1D}	5-HT_{2C}					
	5-HT_{1E}						
	5-HT_{1F}						
Major signalling pathway	G_i/G_0 cAMP↓	G_q\11 IP_3\ DAG↑ PLC↑	ion channel	G_s cAMP↑	5-$HT_{5A:}$ G_s cAMP↓ 5-HT_{5B}: ?	G_s cAMP↑	G_s cAMP↑

2.3. The role of serotonin in depression

The 5-HTT plays a critical role in the regulation of 5-HT neurotransmission, which has been linked to mood and personality disorders (Delgado et al., 1994). Furthermore, serotonin transport has been implicated in the mechanism of widely used antidepressants (Bengel et al., 1997; Lesch, 1997; Lesch et al., 1996). Tricyclics, such as imipramine and amitriptyline, and the selective serotonin reuptake inhibitors (SSRIs) fluvoxamine, fluoxetine, sertraline, citalopram, and paroxetine are widely used in the treatment of depression. Serotonin-norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine inhibits the neuronal uptake of both serotonin and norepinepherine. Tianeptine, an atypical tricyclic antidepressant is enhancing serotonin reuptake.

It has also been described that the incidence of depression is higher among previous ecstasy users (Curran and Travill, 1997; Morgan, 2000; Parrott, 2001; Schifano et al., 1998). One of the most typical symptoms of depression is the modification of sleep patterns (Adrien et al., 1991; Akiskal et al., 1982). The most characteristic alteration is a shortened latency of the first episode of rapid eye movement (REM) sleep, the so-called reduced REM latency, and it has been shown that electroencephalogram (EEG) sleep measurements can yield significant data to aid in differential diagnosis in psychiatry (Akiskal et al., 1997; Kupfer et al., 1978). Strong diagnostic value of REM latency for primary depression versus secondary depression, nonaffective psychiatric disorders and nonpsychiatric controls have been proved, namely, 59% in sensitivity, 81% in specificity, 0% in false positives in controls, and 83% confidence level has been found (Akiskal et al., 1982). Shortened REM latency has also been described in subthreshold depression and so-called ‘borderline’ personality disorders (Akiskal et al., 1997). An association of the s allele of the functional polymorphism of the 5-HTT gene has also been associated with subthreshold depression, and interestingly, this association was primarily carried by the physical symptoms of depression, including sleep disturbance (Gonda et al., 2005).

2.4. The role of serotonin in sleep

5-HT plays a key role in the regulation of sleep, especially REM sleep (Portas et al., 1996). Initiation and duration of REM sleep is modulated by the serotonergic system (Adrien, 2002; Portas et al., 1996). Increase in extracellular 5-HT concentration by 5-HT releasers, 5-HT reuptake blockers or several 5-HT receptor agonists may increase REM latency (Monti and Monti, 1999; Sommerfelt and Ursin, 1991; Ursin, 2002). Physiologic role of 5-HT_2 receptors in sleep regulation have been shown by the use of neutral antagonists (Kitka and Bagdy, 2008). Preclinical studies involving pharmacological depletion or anatomical lesions of 5-HT neurons have generally shown changes in REM and non-rapid eye movement (NREM) sleep. However, most of these preclinical studies involving massive depletions of 5-HT with sleep studies were generally performed shortly after lesioning (Allen et al., 1993; Koella et al., 1968; Touret et al., 1987).

Psychostimulants increase arousal and suppress sleep (McCann and Ricaurte, 2007), therefore a consequence of stimulant abuse is the disruption of normal sleep-wake patterns. MDMA may produce lasting disruptions of sleep and circadian rhythms because it has a neurotoxic potential towards the brain monoaminergic neurons known to modulate normal sleep-wake patterns (Stenberg, 2007).

Although the functional consequences of MDMA-induced 5-HT neurotoxicity are not clear, there have been reports of abstinent MDMA users who developed disorders of mood, anxiety, cognition, impulsivity and sleep following MDMA exposure. Alterations in sleep and circadian rhythm modulation alone can lead to alterations in cognitive function, mood, impulsivity and endocrine function, creating the possibility that alterations in the sleep-wake cycle could play a role in other abnormalities seen in abstinent MDMA users (McCann and Ricaurte, 2007). Therefore, humans who sustain 5-HT injury as a result of their MDMA use may be at a risk for developing altered sleep architecture and disruptions in circadian rhythms.

MDMA abusers have also been reported to suffer from sleep disturbances (Parrott et al., 2000; Williamson et al., 1997) although only a few studies have analyzed the effects of MDMA on sleep patterns in humans and animals in a very controlled fashion (Allen et al., 1993; Biello and Dafters, 2001). Balogh et al. evaluated the effects

of single acute doses (15 mg\kg) of systematically administered MDMA on sleep in Dark Agouti rats (Balogh et al., 2004). Activity and wakefulness increased at least 6 h after MDMA treatment in drug-naïve animals and also in animals that had received a previous single dose of MDMA 21 days earlier. Robust circadian patterns of motor activity and sleep were altered for approximately 5 days after drug treatment, and alterations in motor activity, wakefulness, and "deep" slow wave sleep were still evident 21 days after MDMA administration.

2.5. The role of serotonin in aggression

Serotonin plays a crucial role in the regulation of impulsiveness and aggression (Berman et al., 1997; Morgan, 1998), although other neurotransmitters, notably DA and GABA (gamma-aminobutyric acid) have also been linked to human and animal aggression (Miczek et al., 2002; Nelson and Chiavegatto, 2001). Serotonin release, or high 5-HT levels are generally correlated with low aggressiveness (Nelson and Chiavegatto, 2001; van der Vegt et al., 2003). In contrast, serotonergic dysfunction, as defined by low CSF (cerebrospinal fluid) levels of 5-HT and 5-HIAA (5-hydroxyindole acetic acid), has been associated with increased aggression (Clark and Neumaier, 2001; Scearce-Levie et al., 1999). There is evidence that disorders of central serotonergic neurotransmission, as reflected by low levels of 5-HIAA, are associated with impulsive and aggressive personality traits (Coccaro, 1989; Linnoila et al., 1993).

Acute administration of MDMA has anti-aggressive effects in humans, rats and mice (Curran et al., 2004; Miczek and Haney, 1994), presumably due to serotonin release, but increased impulsiveness and aggression has been found amongst humans who report previous chronic heavy ecstasy use (Bond et al., 2004; Gerra et al., 1998; Morgan, 1998; Parrott et al., 2000). Although it is tempting to speculate that increased aggression in human users is a symptom of MDMA-induced neurotoxicity, the supporting evidence, whilst growing (Curran, 2000; Reneman et al., 2001b; Ricaurte et al., 2000b), is not yet totally compelling. It is possible that symptomatic neurotoxicity may be limited to heavy users, and/ or to particularly susceptible individuals (McCann

and Ricaurte, 1991), in whom vulnerability may be genetically or environmentally determined (Roiser et al., 2005).

2.6. Acute neuropharmacological effects of MDMA in rats

MDMA is a potent indirect monoaminergic agonist and substrate-type 5-HT releasing agent. MDMA acts as a substrate for the 5-HTT, binds to transporter proteins and is subsequently transported into the cytoplasm of nerve terminals (Berger et al., 1992; Rudnick and Wall, 1992). MDMA elevates extracellular 5-HT concentration by a two-pronged mechanism: (1) it promotes efflux of 5-HT by a process of transporter-mediated exchange and (2) it increases cytoplasmic levels of the transmitter by disrupting the storage of 5-HT in vesicles (Rothman and Baumann, 2002). 5-HTTs, in turn, mediate the translocation of drug molecules into the cytoplasm in exchange for 5-HT molecules that flow out into the synaptic cleft. Note that MDMA is also a substrate for the vesicular monoamine transporter (VMAT) present on intracellular vesicle membranes. By disrupting compartmentalization of 5-HT into vesicles, MDMA increases the pool of cytoplasmic transmitter available for drug-induced release (Rothman and Baumann, 2002).

In the rat central nervous system MDMA has characteristic and well-documented biphasic effects upon the serotonergic systems. First, shortly after administration, there is an acute and rapid release of 5-HT (Green et al., 2003; Mechan et al., 2002; Shankaran and Gudelsky, 1998). Second, in the brains of rodents and non-human primates, MDMA causes a long-term reduction in 5-HT and 5-HIAA concentration and 5-HT transporter density. The first phase lasts less than 24 h followed immediately by the second phase which lasts approximately 12 months (or possibly longer) (Schmidt, 1987; Stone et al., 1987b).

Acute effects of MDMA involve several neurotransmitters. Its main effects concern 5-HT, but it also boost dopamine, norepinephrine and acethylcholine (Ach) (Battaglia et al., 1991; Battaglia et al., 1987; Colado et al., 1999; Fitzgerald and Reid, 1990; Johnson et al., 1986; Schmidt et al., 1987; Yamamoto and Spanos, 1988).

MDMA has been shown to increase the release of 5-HT in both in vivo microdyalisis (Gudelsky and Nash, 1996; Mechan et al., 2002; Sabol and Seiden, 1998) and in vitro studies. Under in vitro conditions, the release of 5-HT is increased from brain slices, synaptosomes and cultured neurons following exposure to MDMA (Crespi et al., 1997; Johnson et al., 1986; Schmidt et al., 1987). Data from microdialysis studies also indicate that MDMA enhances the release of 5-HT in vivo in numerous brain regions. The administration of MDMA has been reported to result in a dose dependent increase in the extracellular concentration of 5-HT in the striatum, hippocampus and cortex (Gudelsky and Nash, 1996). Consistent with results from in vitro studies, inhibition of the 5-HT transporter with fluoxetine suppresses MDMA-induced 5-HT release in vivo (Gudelsky and Nash, 1996; Mechan et al., 2002), thereby further supporting the conclusion that MDMA facilitates the transporter-mediated release of 5-HT. MDMA induced 5-HT release is partially Ca^{2+}-dependent (exocytotic-like) and partially Ca^{2+}-independent presumably through the neurotransporter (Crespi et al., 1997).

Under in vitro conditions MDMA has been shown to promote the release of DA from superfused brain slices, as well as to prevent the reuptake of DA into brain synaptosomes (Johnson et al., 1991; Johnson et al., 1986; Schmidt et al., 1987; Steele et al., 1987). Yamamoto and Spanos used in vivo voltammetry to demonstrate that MDMA increases the release of striatal DA in vivo (Yamamoto and Spanos, 1988). Numerous other investigators have confirmed these findings using in vivo microdialysis (Esteban et al., 2001; Gudelsky et al., 1994; Hiramatsu and Cho, 1990; Nash and Nichols, 1991; Sabol and Seiden, 1998).

In vitro MDMA has been shown to induce the release of norepinephrine from brain tissue. Induction of both basal and stimulated [^{3}H]NE release from preloaded rat brain slices was blocked by desipramine (Fitzgerald and Reid, 1990). In a synaptosomal preparation, MDMA induced NE release with similar potency to 5-HT and greater than that for DA (Rothman et al., 2001).

Relatively few studies examined the effects of MDMA on central cholinergic neurons. Fischer et al. observed that MDMA enhances the release of ACh in vitro from striatal slices (Fischer et al., 2000). Acquas et al used in vivo microdialysis to demonstrate that MDMA increases the extracellular concentration of ACh in the

prefrontal cortex and striatum (Acquas et al., 2001). Nair and Gudelsky used in vivo microdialysis and high-performance liquid chromatography with electrochemical detection (HPLC-ED) to assess the stimulatory effect of MDMA on the extracellular concentration of ACh in the prefrontal cortex and dorsal hippocampus of the rat (Nair and Gudelsky, 2006a; Nair and Gudelsky, 2006b).

In the acute phase, one of the most characteristic effects of MDMA is a rapid and pronounced decrease in brain levels of 5-HT a few hours following drug administration. For example, Schmidt demonstrated that both stereoisomers of the drug could produce acute depletion of cortical serotonin measured 3 h after MDMA administration (Schmidt, 1987). Connor et al. have reported a significant depletion in cortical 5-HT concentrations both 30 min and 6 h after MDMA administration (Connor et al., 1998). Acute administration of MDMA in rats also results in a rapid loss of TPH activity in several brain regions (Che et al., 1995; Schmidt and Taylor, 1987). The MDMA-induced decrease in TPH activity is influenced by body temperature. Che et al. demonstrated that lowering body temperature protects against the immediate decrease in TPH activity induced by MDMA (Che et al., 1995).

MDMA competitively inhibited 5-HT catabolism by rat brain MAO-A. Such inhibition reduces the metabolism of 5-HT and dopamine within the nerve terminal and therefore contributes to the increased release of the active neurotransmitter by MDMA (Leonardi and Azmitia, 1994).

Acute treatment with MDMA results in a characteristic behavioural syndrome which includes hyperthermia, hyperactivity, and the serotonin behavioural syndrome (enhanced locomotor activity, reciprocal forepaw treading, head weaving, piloerection, hind limb abduction, proptosis, ataxia and subsequent dose-dependent convulsions and death (Green et al., 2003; Spanos and Yamamoto, 1989).

Data on the effects of MDMA on anxiety in laboratory animals are contradictory**.** Recent experimental findings indicate that acute treatment with MDMA may cause both anxiogenic (Bhattacharya et al., 1998; Gurtman et al., 2002; Lin et al., 1999; Navarro and Maldonado, 1999; Navarro and Maldonado, 2002), and anxiolytic effects (Lin et al., 1999; Morley and McGregor, 2000; Winslow and Insel, 1990) depending upon the test situation (Morley and McGregor, 2000). Furthermore, the dose

range employed (Lin et al., 1999) or the basal anxiety level of the strain used (Green and McGregor, 2002) might also explain the conflicting results observed.

Administration of MDMA produces a significant elevation of rat serum corticosterone and prolactin concentrations 30 min post-injection (Connor et al., 1998; Nash et al., 1988). Aldosterone and renin secretion have also been shown to increase following MDMA treatment of rats (Burns et al., 1996). MDMA and some of its metabolites can stimulate release of both oxytocin and vasopressin, the response being dose-dependent (Forsling et al., 2001; Forsling et al., 2002).

MDMA has cardiac stimulant effects (Gordon et al., 1991), resulting in tachycardia, and arrhythmia and also facilitates vasoconstriction (Fitzgerald and Reid, 1994).

Several studies used a $^{[14C]}$2-deoxyglucose ($^{[14C]}$2-DG) utilization technique to identify brain region specific metabolic changes. Five minutes after a single dose of MDMA (5–30 mg/kg, i.p.), glucose utilization was stimulated in components of the extrapyramidal system and the limbic system showed decrements (Wilkerson and London, 1989). Quate et al. revealed that acute exposure of Dark Agouti rats to a single dose of MDMA (15 mg/kg, i.p.) elicited significant increases in local cerebral glucose utilization that were most pronounced in the motor system and which were uncoupled with acute changes in local cerebral blood flow, suggesting that MDMA might cause acute cerebrovascular dysfunction (Quate et al., 2004).

MDMA binds with less affinity to 5-HT_1, 5-HT_2, α_1, α_2, β-adrenergic, M_1 and M_2 muscarinic, histamine H_1, dopamine D_1 and D_2 and opioid receptors (Morton, 2005). MDMA has weak agonist actions at α_2-adrenoceptors and 5-HT_2 receptors, which might influence its cardiac and pressor effects (Battaglia and De Souza, 1989; Lavelle et al., 1999; Lyon et al., 1986).

2.6.1. Aggregation toxicity

The term “aggregate toxicity” first described by Gunn and Gurd, who found that both behavioural and toxic effects of amphetamine were increased by some factors (Gunn and Gurd, 1940). Later studies noted that toxicity was enhanced by elevated ambient temperature, poor hydration, water deprivation and loud noise (Chance, 1946;

Chance, 1947; Dafters, 1995; Morton et al., 2001). Aggregate toxicity in rodents has been noted for many other drugs, including caffeine, amiphenizole, pipradrol, β-phenylisopropylhydrazine, methylphenidate and methamphetamine (Greenblatt and Osterberg, 1961), isocarboxazide, desmethylimipramine, naloxone, and pethidine (Doggett et al., 1977); morphine (Mohrland and Craigmill, 1980); racemic MDA (Davis and Borne, 1984). MDMA-induced lethality is also increased by grouping animals or housing them in hot conditions (Fantegrossi et al., 2003; Malberg and Seiden, 1998). Investigations into the aggregate toxicity of MDMA may be particularly relevant given the typical "rave" environment in which the drug is often abused by humans. At these all-night dance parties, conditions of crowding and high ambient temperature are common (Green et al., 1995) and may act to potentiate the toxic effects of MDMA. Indeed, an initial report on the lethal effects of MDMA in humans noted that "In almost every case, a recreational dose of the drug had been taken at a dance club or party where crowds danced vigorously" (Randall, 1992).

2.7. Neurotoxicity - long-term neuropharmacological effects of MDMA in rats

2.7.1. Proposed mechanism of MDMA induced neurotoxicity

There are several hypotheses that attempt to explain the mechanism underlying serotonergic neurotoxicity.

2.7.1.1. Role of serotonin

The drug's neurotoxocity is the most important question about its use. The damage has been shown both histologically and biochemically using a variety of experimental techniques in numerous animal species (rats, guinea pigs, squirrel monkeys, cynomolgus monkeys, rhesus monkeys, baboons) and there have been many studies on the mechanisms involved in the neurodegenerative change. Neurotoxicity has been reported in laboratory animals (Commins et al., 1987; Schmidt, 1987; Schmidt and

Taylor, 1987), including axonal changes in serotonergic neurons of nonhuman primates given relatively low dosages (Ricaurte et al., 1988b).

Twenty-four hours after MDMA, tissue 5-HT recovers to normal levels but hydroxylase activity remains diminished. In the long-term phase, which begins within 7 days and lasts for months, MDMA causes a marked depletion of 5-HT and 5-HIAA concentration and loss of 5-HTT binding and function (Battaglia 1988, Scanzello 1993). Most investigators interpret this latter as a neurotoxic effect that involves the serotonergic axons and depletion of terminals (Battaglia et al., 1987; Colado et al., 1993; Green et al., 2003; Ricaurte et al., 2000b; Schmidt, 1987). There is also histological evidence that MDMA produces morphological changes of the 5-HT neurones (Green et al., 1995; Hegadoren et al., 1999; McKenna and Peroutka, 1990; Seiden and Sabol, 1996; Seiden et al., 1993; Steele et al., 1994).

Many drugs used clinically produce effects that are similar to those produced by MDMA. For instance, reserpine causes sustained depletions of brain tissue 5-HT, yet reserpine in not considered a neurotoxin (Carlsson, 1976). Chronic administration of SSRIs, like paroxetine and sertraline, leads to a marked loss of 5-HTT binding and function comparable to MDMA, but these agents are therapeutic drugs rather than neurotoxins (Benmansour et al., 1999; Frazer and Benmansour, 2002). The potent selective serotonin releaser parabromomethamphetamine (V-111) was developed by Knoll et al. and this agent may also be potentially toxic to serotonergic neurons, in a mechanism similar to the action of MDMA (Knoll and Vizi, 1970; Knoll et al., 1970).

The persistent neurotoxic effects on 5-HT neurons following MDMA are similar to those observed following parachloroamphetamine (PCA) in which marked reductions in 5-HT have been observed up to 4 months after a single injection (Kohler et al., 1978; Sanders-Bush and Steranka, 1978). Studies in rats and nonhuman primates show that systemic MDMA administration produces reductions in the levels of 5-HT, 5-HIAA, 5-HT uptake sites or transporters (Battaglia et al., 1988; Lyles and Cadet, 2003; Sprague et al., 1998) and the activity of TPH (Schmidt, 1987; Stone et al., 1987b) which last for months or years after the cessation of drug exposure. Earlier studies also demonstrated these lasting effects on brain 5-HT neurons, using immunocytochemical methods for visualizing 5-HT containing neurons (Axt et al., 1994; Molliver et al., 1990; O'Hearn et al., 1988; Wilson et al., 1989). Many papers reported that [^{3}H]paroxetine binding is

reduced following MDMA administration (Ando et al., 2006; Balogh et al., 2004; Battaglia et al., 1987; Colado and Green, 1995; Ferrington et al., 2006; Scanzello et al., 1993).

MDMA toxicity is affected by doses, routes of administration, as well as by treatment regimens. In addition, age, gender and species used can affect the manifestations of MDMA neurotoxicity. Both the dose and number of injections of MDMA affect the degree of neurotoxicity on 5-HT axons and terminals. Long-term neurotoxic effects of MDMA require either a large single dose (20 mg/kg or more) or several lower doses, typically 5 mg/kg twice daily for 4 consecutive days (Battaglia et al., 1988; Colado et al., 1993; O'Shea et al., 1998; Ricaurte et al., 1988a). O'Shea et al. found that the administration of a single dose of 4 mg\kg MDMA or daily for 4 days had no effect on the serotonergic markers. When this dose was given twice daily for 4 days produced a marked damage (O'Shea et al., 1998).

The neurotoxic effects are evident up to a year after drug administration in rats (Battaglia et al., 1987), and have been observed up to 7 years after drug administration in non-human primates (Hatzidimitriou et al., 1999).

There is also preclinical evidence of regional differences in sensitivity to the neurotoxic effects of MDMA. Areas rich in 5-HT terminals, such as the cerebral cortex show more severe deficits than brain regions containing fibres of passage (e.g. hypothalamus) or cell bodies (brainstem) (Commins et al., 1987; O'Hearn et al., 1988; Steele et al., 1994). In particular, repeated administration of MDMA has been found to produce especially long-lasting degeneration of serotonergic axons, and decreases in brain serotonergic markers in the neocortex, hippocampus, caudate nucleus, putamen and many thalamic nuclei (Aguirre et al., 1997; Fischer et al., 1995; Frederick et al., 1995; Hatzidimitriou et al., 1999; Kleven et al., 1989; Lew et al., 1996; Ricaurte et al., 1992; Sabol et al., 1996).

The results of O'Hearn et al. showed that repeated high-dose MDMA administration has no effect on 5-HT cell bodies in the dorsal raphe, despite a profound loss of 5-HT in forebrain projection areas 2 weeks after MDMA treatment (O'Hearn et al., 1988).

Callahan et al. used anterograde transport of [^{3}H]proline from the raphe nuclei to trace ascending 5-HT axonal pathways in the rat forebrain 3 weeks after MDMA

treatment and observed a marked reductions in the labelled material to various forebrain regions (Callahan et al., 2001). These results were similar to animals previously lesioned with 5,7- dihydroxytryptamine (5,7- DHT), a documented 5-HT neurotoxin. Gartside et al. investigated 5-HT neuronal activity in the dorsal raphe nuclei of rats administered repeated doses of MDMA (Gartside et al., 1996). There were no observable differences in the mean firing rate or regularity of firing of 5-HT neurons between MDMA treated and control animals. Furthermore, electrical stimulation of the dorsal raphe nucleus evoked a threefold increase in cortical and hippocampal dialysate 5-HT levels in both treatment groups. The apparent lack of effect of MDMA administration on electrical activity in the dorsal raphe nucleus is consistent with the observed lack of damage to dorsal raphe nuclei 5-HT cell bodies (Gartside et al., 1996; O'Hearn et al., 1988).

The acute release of 5-HT is proposed to stimulate the 5-HT_{2A} receptor, because 5-HT_{2A} antagonists attenuate MDMA induced neurotoxicity (Nash, 1990; Schmidt et al., 1990a).

2.7.1.2. Role of dopamine

MDMA can selectively destroy serotonergic axons with almost no effect on the dopaminergic system in rats. MDMA increases the extracellular concentration of dopamine in brain regions such as the striatum and nucleus accumbens (Gudelsky and Nash, 1996; Nash, 1990; White et al., 1996; Yamamoto and Spanos, 1988). Several studies hypothesized that the mechanism through which MDMA produces 5-HT neurotoxicity is a dopamine-dependent process in which an MDMA-induced increase in dopamine release results in the uptake of dopamine into 5-HT axon terminals (Sprague and Nichols, 1995) and an induction of oxidative damage (Stone et al., 1989). 5-HT activates 5-HT_{2A} receptors, which enhances dopamine synthesis and release. Blockade of 5-HT_{2A} receptors diminishes MDMA-induced dopamine release (Nash, 1990). 5-HT_2 receptor agonists administered together with MDMA potentiated MDMA-induced DA release (Gudelsky et al., 1994). Moreover, prior administration of 6-hydroxydopamine, which destroys DA terminals, also blocks MDMA neurotoxicity, while pretreatment with L-DOPA (3,4-dihydroxy-L-phenylalanine), a dopamine precursor increases

MDMA neurotoxicity (Schmidt et al., 1990b). Sprague and Nichols suggest that MAO-B metabolizes DA in the 5-HT terminal, producing hydrogen peroxide which could lead to lipid peroxidation and oxidative stress (Sprague and Nichols, 1995).

2.7.1.3. Role of GABA

The role of the GABAergic system in the neurotoxic process appears to be predominantly as a modulator of dopaminergic activity. Colado et al. reported that the GABA agonist chlormethiazole attenuated serotonergic toxicity induced by MDMA (Colado et al., 1993). Furthermore, Yamamoto et al. demonstrated an MDMA-induced increase in extracellular DA coupled with a decrease in extracellular GABA levels in the striatum. GABA might enhance its effects on DA synthesis and release, thus potentiating its neurotoxic effects (Yamamoto et al., 1995).

2.7.1.4. Role of glutamate and nitric oxide

Glutamate, the most abundant excitatory amino acid in the CNS is known to cause neuronal damage and this has led several laboratories to investigate the role of glutamate in MDMA-induced neurotoxicity (Lyles and Cadet, 2003; Sprague and Nichols, 1995).

White et al. demonstrated that locally applied MDMA has inhibitory effects on glutamate-evoked neuronal firing in the nucleus accumbens and suggested that the inhibition is mediated by increased extracellular dopamine and serotonin (White et al., 1994).

One study showed that the coadministration of the NMDA-receptor antagonist, MK-801 and MDMA caused a dose-related attenuation of the 5-HT depletion 7 days after treatment (Colado et al., 1993; Finnegan and Taraska, 1996). In contrast, another study found that coadministration of MK-801 attenuated only the methamphetamine-induced decrease in TPH activity, but did not alter the effects of MDMA (Johnson et al., 1989)

The role of NO (nitric oxide) in the neurotoxicity of MDMA has also been studied in rats. Taraska et al. concluded that NO plays little or no role in the toxic

mechanism of action of MDMA because N^Gnitro-L-arginine methyl ester (L-NAME) was the only NO synthase inhibitor that antagonized the dopamine- and serotonin-depleting effects of MDMA and induced hypothermic response (Taraska and Finnegan, 1997). Two other NOS inhibitors, N^G-monomethyl-L-arginine and N^G-nitro-L-arginine neither blocked the neurotoxic effects of MDMA nor caused hypothermia. However, another study (Simantov and Tauber, 1997) found that MDMA can cause death of human serotonergic cells and that these cells could be protected by L-NAME in the culture.

2.7.1.5. Role of metabolites

Previous studies proposed that MDMA metabolites are the ultimate pathway of serotonergic neurotoxicity. Schmidt and Taylor reported that acute cerebroventricular injections of MDMA into the rat brain do not produce 5-HT axon destruction, therefore any toxic metabolite produced would most likely be formed in the periphery (Schmidt and Taylor, 1988).

Subcutan injection of MDA, MDMA, HMA (4-hydroxy-3-methoxyamphetamine) and α-methyldopamine decreased the concentration of 5-HT in the frontal cortex (Yeh and Hsu, 1991). In contrast, systemic administration of the major metabolites of MDA, HHA (3,4- dihydroxyamphetamine) and of MDMA, DHMA (3,4-dihydroxymethamphetamine) did not produce neurotoxicity (McKenna and Peroutka, 1990). DHMA is metabolized to quinone-like structures that were thought to be involved in MDMA-induced neurotoxicity (Hiramatsu et al., 1990). MDMA metabolites generate free radicals through redox cycling and these reactive species could inactivate TPH and cause damage to protein and lipid components of the neuron terminal. Catechols, hydroquinones and quinines can undergo spontaneous oxidation by oxygen, to generate superoxides and hydrogen peroxide and these by-products could lead to membrane damage and 5-HT terminal loss (Colado and Green, 1995; Paris and Cunningham, 1992).

It has been supported that both oxidative and bioenergetic stress can underlie the mechanism of MDMA induced neurotoxicity (Darvesh and Gudelsky, 2005; Gudelsky and Yamamoto, 2003). Microdyalisis studies showing that MDMA induces oxidative

stress include the findings that MDMA increases the formation of reactive oxygen species (e.g. hydroxyl radicals) and nitrogen species (e.g. nitric oxide) (Colado et al., 1997; Shankaran and Gudelsky, 1999). Shankaran et al. demonstrated that concomitant treatment of rats with ascorbic acid and a neurotoxic regimen of MDMA not only prevents MDMA induced depletion of brain 5-HT but also the loss in ability of MDMA to evoke 5-HT release (Shankaran et al., 2001).

It has also been suggested that 5-HT or a metabolite of 5-HT (e.g. 5,6- or 5,7-dihydroxytriptamine) may be responsible for the toxicity. Blockade of 5-HT uptake by fluoxetine or citalopram antagonizes MDMA induced 5-HT depletion and facilitates the recovery of TPH activity (Lyles and Cadet, 2003; Schmidt, 1987; Schmidt and Taylor, 1987).

2.7.1.6. Role of hyperthermia

Several laboratories have shown that MDMA produce elevated body temperature of rats at ambient temperature (Nash et al., 1988). Fatal hyperthermia is associated with MDMA use in humans (Chadwick et al., 1991; Dar and McBrien, 1996).

Malberg et al. has found a close correlation between temperature response and degree of neurotoxicity (Malberg and Seiden, 1998). Maintenance of animals at low ambient temperature (10^{o}C) before and after treatment with MDMA prevents the hyperthermic response, producing hypothermia in some cases, and either attenuates or eliminates MDMA-induced neurotoxicity (Broening et al., 1995; Schmidt et al., 1990a). At elevated room temperatures (26-33^{o}C), both the hyperthermic response and the neurotoxicity are potentiated (Broening et al., 1995; Sanchez et al., 2004). Despite this close relationship between acute hyperthermia and subsequent neurotoxicity the exact role of hyperthermia in the development of the neurotoxic response is difficult to define since it is possible to observe neurotoxicity also in the absence of hyperthermia (Broening et al., 1995; O'Shea et al., 1998). In contrast, haloperidol, which prevents MDMA-induced hyperthermia (Colado et al., 1999), pentobarbitone, NMDA antagonists such as dizocilpine or the 5-HT$_{2A}$ receptor antagonist ketanserin which produce hypothermia (Colado et al., 1999; Farfel and Seiden, 1995; Malberg et al.,

1996) all appear to have no specific protective effect against MDMA-induced neurotoxicity. Furthermore, a few agents e.g. fluoxetine and clomethiazole have been found to protect against the neurotoxicity of MDMA with no modification of temperature (Colado et al., 1999; Colado et al., 1997; Sanchez et al., 2001). Several studies conclude that neurotoxicity and hyperthermia are separable (Broening et al., 1995; Colado et al., 1997; O'Shea et al., 1998). For example, MDMA administration produced a hyperthermic response after every dose when the drug was given twice weekly but no neurodegeneration was observed. Taken together, this evidence indicates that hyperthermia has an important role but is not an essential factor in MDMA-induced neurotoxicity.

2.7.2. Neuronal recovery

Björklund et al. reported that the serotonergic neurons show high capacity for axonal sprouting and regeneration in the mature mammalian brain (Björklund et al., 1981). Several studies demonstrated that the administration of 5,6-DHT, a neurotoxic analogue of serotonin leads to axotomy and widespread terminal degeneration of serotonergic neurons. The lesioned serotonergic neurons have the capacity to regrow and re-establish some of their original connections (Björklund et al., 1973; Björklund and Wiklund, 1980; Nobin et al., 1973; Wiklund and Bjorklund, 1980).

Previous studies report 5-HT neuronal recovery after PCA (Sanders-Bush et al., 1972) and fenfluramine (Harvey and McMaster, 1975; Kleven et al., 1988). In contrast, two studies suggest that 5-HT recovery in rats exposed to toxic amphetamine derivatives may not be sustained. Zaczek et al. found an initial recovery of 5-HT neuronal markers after fenfluramine treatment, but recovery is not maintained because severe 5-HT deficits are apparent 6 to 8 months after drug administration (Zaczek et al., 1990). Mamounas et al. also demonstrated an initial regenerative sprouting of 5-HT axons after PCA injury, 6-12 months after PCA 5-HT axons were reduced in number and abnormal (Mamounas et al., 1991).

Rats treated with MDMA show partial recovery of tissue 5-HT content, and complete recovery of [^{3}H]paroxetine-labeled 5-HT uptake sites 1 year after drug administration (Battaglia et al., 1988; De Souza and Battaglia, 1989; De Souza et al.,

1990). MDMA-induced reductions in 5-HT levels and 5-HTT binding eventually recover (Battaglia et al., 1988; Scanzello et al., 1993), suggesting the possibility that 5-HT terminals are not destroyed. Similarly, Molliver et al. observed a regenerative sprouting of 5-HT axons during 6 to 8 months after drug treatment (Molliver et al., 1990; Molliver et al., 1989).

Scanzello et al. observed a recovery of 5-HT content 16 weeks after MDMA treatment in some brain areas (e.g. hypothalamus, striatum) and at 32 weeks the recovery was nearly complete in most brain regions (Scanzello et al., 1993). [^{3}H]paroxetine-binding values in hippocampus were 29% below control values at 52 weeks post-treatment. Recovery over the 52 week interval was noted for all depleted regions but the rate and degree of recovery was found to be region dependent (Lew et al., 1996; Sabol et al., 1996).

52 weeks after MDMA treatment there is evidence of a lasting reorganisation of ascending 5-HT axon projections, with projections to distant forebrain sites (e.g. dorsal neocortex) exhibiting little or no evidence of recovery, while projections to some more proximal targets (e.g. hypothalamus) recover fully or in excess (Fischer et al., 1995). These results indicate that altered reinnervation patterns develop much more frequently in MDMA-treated primates than in MDMA-treated rodents. Other studies also observed that in primates MDMA-induced 5-HT neural damage is persistent, and possibly permanent (Insel et al., 1989; Ricaurte et al., 1992). MDMA-treated monkeys show evidence of a "reorganization" of ascending 5-HT projections that is remarkably similar to the "pruning effect" observed after a variety of neuron lesioning techniques, including some using 5,6- and 5,7-DHT (Björklund et al., 1981; Frankfurt and Azmitia, 1984; Wiklund et al., 1978).

2.8. Problems in relating animal and human data - interspecies scaling, dose, age and gender differences

The extrapolation of animal data to human recreational use is very difficult and several critical factors must be considered, including species differences, route and schedule of drug administration and drug dosages. A major point of controversy is related to the relevance of MDMA doses administered to rats when compared to doses

taken by humans (Cole and Sumnall, 2003). The pharmacokinetics of the drug in rats and humans may be similar. For example a dose of 1.5 mg/kg MDMA administered to humans resulted in a peak plasma concentration of 331 ng/ml. Extrapolation from data obtained with a dose of 10 mg/kg administered to female Dark Agouti rats suggests that administration of a dose of 1.5 mg\kg would produce a very similar plasma concentration. There is a view among some recreational ecstasy users that the doses required to induce damage in experimental animals are so high that they have little relevance to those taken by humans and that the drug is therefore „safe". This view is weakened by the observation that a single dose of MDMA of 10 mg/kg produced drug levels similar to those involved in acute adverse reactions and that this dose caused considerable neurotoxic damage to 5-HT nerve terminals (Colado and Green, 1995; Green et al., 2003). It is undoubtedly true that many experimental studies have used high doses, and it now appears that different strains exhibit a different sensitivity to the drug (Malpass et al., 1999) and the long-term neurotoxic effects of MDMA.

The most obvious example is the Dark Agouti strain, which requires a single dose (10–15 mg/ kg) of MDMA to produce a clear 30 to 50% or greater loss in cerebral 5-HT content (Colado and Green, 1995; O'Shea et al., 1998). This contrasts with the several doses of MDMA, often a cumulative dose of 80 mg/kg within a few hours, that are usually required to produce a similar loss in Sprague-Dawley, Hooded Lister and Wistar rats (Aguirre et al., 1998; Colado et al., 1993; Shankaran and Gudelsky, 1999).

The dose required to induce neurotoxicity is strain-dependent. Humans typically use MDMA orally, and although they may use up to 10 dosages per night, usually do not use it during 4 consecutive days. The levels of neurotoxicity produced by oral versus parenteral MDMA do not differ (Finnegan et al., 1988). However, De Souza et al. reported that in rats, oral administration was less effective than intraperitoneal (De Souza et al., 1997).

The neurodegenerative effects of MDMA on 5-HT neurons exhibited some species specificity. The mouse is the one animal species that is relatively resistant to MDMA-induced 5-HT injury, but at high doses mice also develop persistent loss in brain 5-HT markers (Stone et al., 1987a). MDMA is neurotoxic to DA axons rather than 5-HT axons in mice (Cadet et al., 1995; Cadet et al., 2001; Colado et al., 2004). Cadet et

al. reported that a high dose of MDMA (50 mg\kg) produced dopamine depletion in the striatum of non-transgenic mice (Cadet et al., 1995).

MDMA is self-administered by both rhesus monkeys and baboons suggesting that MDMA may have a high abuse potential in man. Long lasting effects similar to those observed in rats have also been reported in nonhuman primates. In nonhuman primates MDMA causes dose-dependent reductions in 5-HT in the cortex, caudate nucleus, putamen, hippocampus, hypothalamus and the thalamus. Reduced 5-HT level was evident for up to 7 years following MDMA administration (Hatzidimitriou et al., 1999). Immunohistochemical studies have also revealed marked decreases in axon density in the forebrain of nonhuman primates treated with MDMA.

Studies also reported that altered reinnervation patterns develop much more frequently in MDMA-treated primates than in MDMA-treated rodents. Baboons treated with MDMA (5 mg/kg MDMA, twice daily for 4 consecutive days) also show marked and prolonged decreases in 5-HT transporter density measured by PET with radioligand (McN-5652), which selectively labels the 5-HT transporter (Scheffel et al., 1998). In agreement with the results of Fischer et al., PET scans 9 and 13 months post-MDMA showed regional differences in the apparent recovery of the 5-HT transporter, with increases in some brain regions (e.g. hypothalamus) and persistent decreases in others (e.g. neocortex) (Fischer et al., 1995).

Dosages of MDMA that produce neurotoxicity in rodents are higher than those needed to produce neurotoxicity in primates (Battaglia et al., 1987; Ricaurte et al., 1988a; Stone et al., 1986). The observation that smaller animal species require higher doses of the drug to achieve equivalent drug effects is predicted by the principles of interspecies scaling. The concept of interspecies scaling is based upon shared biochemical mechanisms among eukaryotic cells (e.g. aerobic respiration), and it was initially developed to describe variations in basal metabolic rate (BMR) between animal species of different sizes (White and Seymour, 2005). This method utilizes known relations between body mass/surface area and accounts for differences in drug clearance. Similarly, the same technique can be applied to predict dosages of MDMA that would be neurotoxic in humans. It is known that the neurotoxic dose in 1 kg squirrel monkeys is 5 mg/kg and the equivalent dose in humans is found to be 1,28 mg/kg or approximately 96 mg in a 75 kg individual. The typical amount of MDMA

abused by humans is one or two tablets of 80-150 mg or 1-3 mg/kg administered orally (Green et al., 2003; Schifano, 2004). This dose falls squarely in the neurotoxic range predicted by the interspecies scaling method.

It is also important to consider the influence of age and sex differences on the effects of MDMA. The role of age is known to cause variability in the sensitivity of animals and humans to MDMA (Easton and Marsden, 2006). Principal users are adolescents and young adults. Coincidentally, these are also peak reproductive ages. There are few clinical reports on the effects on offspring development after intrauterine exposure. One group has suggested that there may be an increase in congenital malformations after prenatal MDMA exposure, including defects of the heart and feet (McElhatton et al., 1999), but no clinical studies have appeared on possible effects on brain development and function.

It has been demonstarted that the persistent effects of MDMA on the serotonergic system in the rat are modulated by developmental age (Broening et al., 1994). Results from animal studies suggest that fetal and neonatal rat brains are not vulnerable to MDMA-induced serotonergic neurotoxicity (Allen et al., 1993; Broening et al., 1994; Colado et al., 1997; Kelly et al., 2002), however other studies have found effects (Koprich et al., 2003; Won et al., 2002). For example, Winslow and Insel found that single or repeated doses of MDMA administered on P1–4 result in decreased ultrasonic calling in rat pups and reduced cortical 5-HT and 5-HTT binding on P21 (Winslow and Insel, 1990). Meyer and Ali found that rats exposed to MDMA on P1– 4 and evaluated at P25 or P60 showed reduced hippocampal 5-HT concentrations and 5-HTT binding at P25, and hippocampal 5-HTT binding remained reduced at P60 (Meyer and Ali, 2002).

MDMA administration does not produce long-term 5-HT loss in immature rats (PND 10) when compared to more mature rats (PND 40, 70) where damage does occur (Broening et al., 1994). Piper and Meyer demonstrated that intermittent MDMA exposure to rats during the adolescent period of development can produce memory deficits and reduce anxiety in the absence of severe serotonergic damage (Piper and Meyer, 2004). Additionally, long-term deficits in reference memory are produced following MDMA exposure to rats of PND 11–20 (Vorhees et al., 2004) and a

combination of early and late exposure of MDMA in rats can produce disruption of spatial acquisition and reversal learning (Cohen et al., 2005).

There are very little available data in humans which compare the extent of behavioural, biochemical or physiological effects between adolescent and adult user groups of MDMA. Deficits in verbal and visual memory have been most frequently observed in adults (Gouzoulis-Mayfrank et al., 2000; Morgan, 1999; Parrott et al., 1998). Jacobsen et al. found that MDMA use in adolescence may be associated with cognitive impairments and dysfunction of inhibitory circuits within the hippocampus. These data indicate that similar behavioural changes occur in both age group (Jacobsen et al., 2004).

There have been few studies which examine the role of gender differences related to the effects of MDMA. Although more men than women are involved in alcohol, tobacco or illicit drug use, there is growing evidence based on animal and clinical studies that females are more susceptible to the effects of drugs of abuse. Two recent behavioral studies report that females show enhanced locomotion, less startle response, and comparable effects in the plus maze (Bubenikova et al., 2005; Palenicek et al., 2005) in Wistar rats after MDMA administration. Female Sprague-Dawley rats show lower brain concentrations of MDA compared to males, suggesting a slower metabolism of MDMA to MDA in females (Chu et al., 1996). Fitzgerald et al. showed a higher level of MDMA in the plasma of female rats after a single drug injection (Fitzgerald et al., 1989). Walker et al. found that MDMA elicited much more robust locomotor activation in female than in male rats, but caused similar serotonin depletion and persistent anxiety-like behaviors 2 weeks after the end of treatment (Walker et al., 2007). A previous study in rodents has shown that male and female rats have comparable brain levels of MDMA after acute treatment (Chu et al., 1996). Similar results of comparable 5-HT depletion in males and females were also reported by Slikker et al. (Slikker et al., 1989). Colado et al. demonstrated that female Dark Agouti rats are more susceptible to the acute hyperthermic effects of MDMA (Colado et al., 1995). The concentration of 5-HT and 5-HIAA in the cortex and hippocampus of male and female rats were similar 7 days after injection of MDMA. Female Dark Agouti rats having markedly decreased debrisoquine 4-hydroxylase activity than males (Gonzalez et al., 1987). However, several investigators reported that in mice and rats, MDMA is

more toxic in males than females (Cadet et al., 1994; Koenig et al., 2005; Miller and O'Callaghan, 1995). Koenig et al. observed that the male Long–Evans rats appear to be more sensitive to locomotor and hyperpyretic effects of MDMA. Presumably the effects of MDMA in rats depends on the used rat strain (Koenig et al., 2005).

Veryheyden et al. observed that women are more susceptible than men to mid-week low mood following weekend use of MDMA (Verheyden et al., 2002). Liechti et al. reported greater subjective responses to MDMA in women than in men, including greater thought disturbances, perceptual changes, and fear of loss of body control. In contrast, men experience greater increases in blood pressure (Liechti et al., 2001). Other studies using SPECT or PET also indicate that women might be more susceptible than men (Buchert et al., 2004; Reneman et al., 2001a). Biochemical evidence supports this suggestion as analysis of CSF data revealed that reductions of 5-HIAA CSF were greater in females than in males (46% vs 20%) (McCann et al., 1994). Buchert et al. found reduction of serotonin transporter availability in current MDMA users and this was more pronounced in female than in male subjects (Buchert et al., 2004). Schifano reports that the MDMA lethality ratio between males and females is 4:1 (Schifano, 2004). Although these reports suggest that the effects of MDMA are not equivalent in men and women, they could be related to differences in innate hormonal profiles, volume and morphology of certain brain structures, or monoaminergic neurotransmission.

2.9. Metabolism of MDMA

In humans, MDMA is metabolised principally by cytochrome P450 (CYP) 2D6 or debrisoquine hydroxylase (Tucker et al., 1994). Because of the polymorphic variants of this isoenzyme, 5- 9% of the Caucasian population is considered to be a poor metabolizer phenotype (Gonzalez and Meyer, 1991). There is evidence that “poor metabolizers” of debrisoquine have lower metabolic capacity and higher plasma levels of MDMA (de la Torre et al., 2005). In rats, MDMA is metabolized by an analogous isoenzyme, CYP2D1. The Dark Agouti rat strain possesses decreased microsomal CYP2D1 isoenzyme activity, and thus, the Dark Agouti rats are thought to be similar to vulnerable human users, the “poor metabolizers”. The Dark Agouti rat is a useful

experimental tool to model MDMA exposures that are pertinent to the human experience of the drug (Balogh et al., 2004; Kirilly et al., 2006; O'Shea et al., 1998; Quate et al., 2004). This rat strain provides a model of a genetically- defined human sub- population in which clinical complications may be more likely to occur.

MDMA displays nonlinear kinetics (de la Torre et al., 2000), which has implications for the acute toxic response to the drug when a high dose, or more than one dose, is taken in a single session (Farre et al., 2004). Approximately 8% of the MDMA dose is excreted unchanged in rats but this rises to 50% in humans (de la Torre et al., 2000).

Ecstasy tablets ingested by humans contain a racemic mixture of (+) and (-) isomers of MDMA, and both stereoisomers are known to be bioactive (Johnson et al., 1986; Schmidt et al., 1987).

Several pathways are involved in MDMA metabolism in the rat: *N*-demethylation, *O*-dealkylation, deamination, and conjugation (*O*-methylation, *O*-glucoronidation, and/or *O*-sulfation), resulting in the formation of 14 in vivo metabolites (Lim and Foltz, 1988; Lim and Foltz, 1991a; Lim and Foltz, 1991b). Firstly, MDMA is metabolized into MDA by *N*-demethylation, DHMA by demethylenation, and 2-hydroxy-4,5-(methylenedioxy)methamphetamine by ring hydroxylation (Cho et al., 1990; Lim and Foltz, 1988; Lim and Foltz, 1991a; Tucker et al., 1994). These undergo further metabolism (possibly via catechol-O-methyl transferase mediated reactions) to form HMMA (4-hydroxy-3-methoxy-methamphetamine) and HMA, respectively.

2.10. Human studies

MDMA exhibits both psychostimulant and hallucinogenic properties (Shulgin, 1986). MDMA is often described as an entactogen producing a sense of touching within (Gudelsky and Yamamoto, 2008; Nichols, 1986). The subjective effects of MDMA in humans include a sense of well being, connectedness and diminished aggression (Davison and Parrott, 1997; McDowell and Kleber, 1994). MDMA use is generally associated with happy young people enjoying themselves in the social situation of dance clubs or “raves”.

2.10.1. Acute pharmacological effects in humans

MDMA is rapidly absorbed following oral administration. The onset of effects can take 20 to 60 min to occur, reaches its peak in 60 to 90 min after ingestion, and the primary effects last for 3 to 5 h. Acute adverse effects are related to the dose ingested, the frequency of dosing, the ingestion of other drugs (e.g. alcohol, nicotine, amphetamine, cocaine, ketamine, and many other psychoactive drugs (Huxster et al., 2006; Soar et al., 2006; Thomasius et al., 2006) and the environmental conditions in which the user was present.

The acute psychological effects of MDMA include feelings of euphoria, increased closeness to others, elevated self-confidence, heightened arousal and sensory awareness (Downing, 1986; Greer and Tolbert, 1986; Liechti and Vollenweider, 2000), increased sensory sensitivity (Cohen, 1995; Peroutka et al., 1988; Solowij et al., 1992). More recent studies reported several acute side effects e.g. moderate derealisation and depersonalisation, cognitive disturbances, elevated anxiety, tachycardia, panic attacks (Cohen, 1995; Downing, 1986; Liechti and Vollenweider, 2000; Liester et al., 1992; Whitaker-Azmitia and Aronson, 1989). Prospective and cross-sectional studies have shown evidence for psychiatric and/or psychobiological problems prior to drug use (Huxster et al., 2006; Soar et al., 2006; Thomasius et al., 2006).

Acute adverse physical effects of MDMA include suppressed appetite, bruxism, papillary dilatation, trismus (jaw-clenching), dry mouth, palpitation, nausea, impaired judgement and gait (ataxia). Subjects also exhibit symptoms of sympathetic arousal including tremors and tachycardia (Downing, 1986; Green et al., 2003; McCann et al., 1996; Peroutka et al., 1988).

After-effects may be quite pronounced, sometimes lasting 24- to 48 hours. The most dramatic “hangover” effect is a sometimes profound anhedonia, depression, irritability, difficulty in concentrating, headache, muscle aches, fatigue, but the users may experience lethargy, anorexia, and decreased motivation (Cohen, 1995; Davison and Parrott, 1997; Peroutka et al., 1988; Solowij et al., 1992).

Serious adverse effects of MDMA intoxication include cardiac arrhythmias, hypertension, hyperthermia (body temperatures over 43^{o}C), acute renal failure, rhabdomyolisis, disseminated intravascular coagulation, serotonin syndrome,

hyponatremia, liver problems (centrilobular necrosis and microvascular steatosis, acute cholestatic hepatitis) (Devlin and Henry, 2008; Milroy et al., 1996), seizures, coma, and, in rare cases, death. MDMA impairs temperature control, so that in the hot and crowded conditions at raves many dancers become overheated or hyperthermic (Dafters, 1994).

MDMA deaths are comparatively rare, but unfortunately they show a year-by year increase, which may be facilitated by the decreasing cost of MDMA (Schifano et al., 2006).

2.10.2. Long-term pharmacological effects in humans

The most important question is whether MDMA abuse causes neurotoxic damage in humans. There is a growing consensus that MDMA might indeed be toxic to humans. The neurotoxic potential of MDMA in humans has been evaluated indirectly by measuring the concentration of 5-HIAA in cerebrospinal fluid in recreational ecstasy users. The earliest study employing this technique failed to find evidence of reduced levels of 5-HIAA in recreational users (Peroutka, 1987). However, other studies (McCann et al., 1999; McCann et al., 1994; Ricaurte et al., 1990) reported significantly lower levels of CSF 5-HIAA in recreational ecstasy users compared to polydrug users who had never used ecstasy. There were no changes in the levels of the DA metabolite homovanillic acid nor in the concentration of the NE metabolite 3-methoxy-4-hydroxy-phenylglycol (MHPG). Kish et al. demonstrated severe depletion (50-80%) of striatal 5-HT and 5-HIAA in the brain of a 26-year-old male who had taken MDMA regularly for 9 years (Kish et al., 2000).

Accumulating evidence indicates that chronic, heavy, recreational use of ecstasy is associated with sleep disorders, depressed mood, persistent elevation of anxiety, impulsiveness and hostility, and selective impairment of episodic memory, working memory and attention, which can last for months after cessation of drug intake (Morgan, 2000; Parrott, 2002; Parrott et al., 2002).

Recently, more direct evidence that MDMA produces long-term neurotoxic effects on brain 5-HT systems has emerged from neuroimaging studies: decrease in 5-HTT detected by positron emission tomography (PET) and single photon emission computed tomography (SPECT), and a decrease in the neurometabolite NAA, a marker

for non-specific neuronal loss, detected by proton magnetic resonance spectroscopy (^{1}H-MRS) (Reneman et al., 2006). Daumann et al. found no differences in neocortical regions, only a tendency towards lower NAA/Cr ratios in the hippocampus of MDMA users, and therefore concluded that 1H-MRS is a less sensitive marker of potential neurotoxic damage in ecstasy users (Daumann et al., 2004).

McCann et al. used positron emission tomography (PET) with the first- and second- generation radioligand, carbon-11-labelled McN-5652 and DASB, which selectively labels the 5-HT transporter. MDMA users showed decreased global and regional brain 5-HT transporter binding with both PET ligands (McCann et al., 1998; McCann et al., 2005). Ricaurte et al. observed decreased 5-HT transporter binding which positively correlated with the extent of previous MDMA use (Ricaurte et al., 2000a). Buchert et al. observed similar changes and found that women are more susceptible than men to MDMA-induced alterations of the serotonergic system (Buchert et al., 2003; Buchert et al., 2004).

Imaging studies used SPECT with the 5-HT transporter radioligand ($^{[123I]}$ b-CIT) to investigate heavy ecstasy users and controls. The ecstasy users showed a widespread reduction of 5-HT transporter binding (Haddad et al., 2002; Reneman et al., 2001b; Semple et al., 1999).

The status of 5-HT markers in MDMA users has also been assessed with SPECT using $^{[123I]}$-I-R91150 a radioligand, which binds with a high affinity to 5-HT_{2A} receptors. 5-HT_{2A} receptor densities appeared to be significantly increased in the occipital cortex of ecstasy users compared to controls, possibly as a compensatory response to 5-HT depletion (Reneman et al., 2000).

There are a number of case reports of chronic psychiatric symptoms following regular use of the drug including reports of depersonalisation (McGuire et al., 1994), obsessive-compulsive symptoms (Marchesi et al., 2007), flashbacks (Creighton et al., 1991; McGuire and Fahy, 1992; Schifano and Magni, 1994), panic attacks (Cohen, 1996; McCann and Ricaurte, 1991; McGuire et al., 1994; Schifano and Magni, 1994; Series et al., 1994), psychosis (Creighton et al., 1991; McCann and Ricaurte, 1991; McGuire and Fahy, 1991; McGuire et al., 1994; Schifano, 1991) and depression (Benazzi and Mazzoli, 1991; McCann and Ricaurte, 1991; McGuire et al., 1994; Schifano and Magni, 1994). Other studies have also shown that MDMA abuse can

negatively impact the performance of memory and psychomotor tasks (Gouzoulis-Mayfrank et al., 2000; Morgan, 1999; Wareing et al., 2000). Memory was the first area of cognition shown to be impaired in recreational ecstasy users (Krystal et al., 1992) and this had been confirmed in several laboratories (McCann et al., 2000; Parrott et al., 2000). Other psychobiological deficits include eating disorders (Curran and Robjant, 2006), impaired social and emotional judgement (Reay et al., 2006), and selective changes in auditory evoked potentials (Daumann et al., 2006).

Clinical studies designed to critically evaluate the long-term effects of MDMA are hampered by a number of factors, including comorbid psychopathology and polydrug abuse among MDMA users. There are many variables that could not be controlled, e.g. dose and purity of MDMA, number of times MDMA was used, set and setting, and the time between interview and the last use of MDMA. Animal models afford the unique opportunity to evaluate the effects of MDMA without many complicating factors. The existing preclinical data strongly suggest that MDMA can produce neurotoxic damage in the brain. Therefore, any prospective clinical study involving MDMA administration is constrained by ethical considerations. Even administration of low doses of MDMA (1.7 mg/kg) has been publicly questioned and discussed (McCann et al., 2001; Vollenweider et al., 2001). All studies can be criticized for the fact that accurate information on both the doses taken and the duration of drug use is not available. This is because the drug has been synthesized and supplied illicitly and most subjects are unreliable sources of information on use. In addition, many subjects are poly-drug users so that it cannot be stated unequivocally that any event seen results solely from MDMA use. Alternatively, the problem may relate to the combination of MDMA with another recreational compound. This problem is compounded by the fact that a significant percentage of ecstasy tablets contain psychoactive compounds other than MDMA.

With regard to the possible therapeutic use, one group in particular has long advocated a trial for the use of MDMA in post-traumatic stress disorder (Bouso et al., 2008), and other trials for the use of MDMA to help rape or cancer victims have also been proposed.

3. OBJECTIVES

The recreational drug ecstasy, 3,4-methylenedioxymethamphetamine (MDMA), has been found to selectively damage brain serotonin neurons in experimental animals, and probably in human MDMA users**,** but detailed morphometric analyses and parallel functional measures during damage and recovery are missing. We specifically emphasized the long-term morphological effects of a single dose of MDMA from the brainstem up to the cerebral cortex. We hypothesized that long-lasting damage of the serotonergic system will be manifested in changes of several sleep parameters. Furthermore, we investigated the effects of MDMA on aggressive behaviour. These studies were carried out in Dark Agouti rats, a special strain that may serve as a genetic model for poor or moderate metabolizer ecstasy users.

The main objectives in our studies were:

1. How does 5-HTT mRNA expression change in the different raphe nuclei during 6 months after MDMA treatment?
2. Can differences be observed in the changes concerning dorsal and median raphe nuclei?
3. How does 5-HTT-immunoreactive (IR) fibre density change in the different brain areas?
4. Do the examined brain areas show differences in sensitivity after MDMA treatment?
5. How much regeneration can be observed 6 months after MDMA treatment?
6. How do sleep parameters change after MDMA treatment?
7. What sleep parameters change similarly and the opposite way compared to what has previously been observed during depression?
8. How does MDMA treatment alter agressive behaviour?

4. MATERIALS AND METHODS

4.1. Animals

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National Institutes of Health "Principles of laboratory animal care" (NIH Publications No. 85-23, revised 1985). In addition, specific national laws were adhered to (Hungarian Governmental Regulation about animal studies, December 31, 1998 and United Kingdom Animals (Scientific Procedures) Act, 1986) and permission was obtained from local ethical committees (22.1/2655/001/2007). Six to seven-week old male Dark Agouti rats (Harlan, Olac Ltd, Shaw's Farm, Blackthorn, Bicester, Oxon, UK, aged 4-5 weeks and weighing 50-80 g upon arrival) were used in the experiments. The animals (4 per cage) were kept under controlled environmental conditions (temperature at 21±1 ^{o}C, and a 12 hour light-dark cycle starting at 10.00 a.m), with standard rat chow and water freely available. To establish the resident status of the rats, these animals were housed singly for the final two weeks prior to the test.

4.2. Drugs

(±)3,4-methylenedioxymethamphetamine hydrochloride (MDMA, certified reference compound, purity >99.5%) was provided by Sanofi-Synthelabo-Chinoin, Budapest, Hungary. The drug was dissolved in 0.9% NaCl at dose equivalent to 15 mg/kg free base and was injected intraperitoneally (i.p.) in a volume of 1 ml/kg. Control animals received an injection of 0.9% NaCl (SAL, saline) in a volume of 1 ml/kg.

4.3. Treatment protocol

MDMA or saline pretreatment was administered at the age of 7 weeks and the resident-intruder test was carried out 3 weeks later when the resident animals were 10 weeks old (weight 215±2.50 g) and the intruders were 5-6 weeks old (weight

80.65±1.35 g). Resident rats were pretreated with MDMA (15 mg/kg) or saline on a single occasion, 3 weeks prior to the acute drug administration (MDMA), which occurred twenty minutes prior to the introduction of the intruder to the home cage of the resident (Fish et al., 1999). Treatment groups consisted of 7 animals in the resident-intruder test. Each rat was tested only once.

To measure the time course of serotonin transporter mRNA expression after MDMA treatment, *in situ* hybridization histochemistry was performed 3, 7, 21 and 180 days after drug treatment (n = 48: 6 animals in each control and treated group at the 4 time points). Paralelly, in separate subgroups of rats, the density of serotonergic fibres was assessed using 5-HTT immunohistochemistry (n = 5- 6 per groups). Separate subgroups of animals were used for vigilance studies (n = 7- 9 per groups) and for the behavioural studies (n= 7).

4.4. Experimental procedures

4.4.1. In situ hybridization histochemistry

4.4.1.1. Section preparation

The animals were decapitated 3, 7, 21 and 180 days after MDMA treatment. The brains were removed, frozen on dry ice and stored at -70^{o}C. Coronal serial sections (12μm) were cut throughout the lower brainstem at -20^{o}C using cryostat (Reichert-Jung Cryocut 1800) and thaw-mounted onto silanized slides, dried on a slide warmer and stored at -70^{o}C until hybridization and further quantitative analysis.

4.4.1.2. mRNA probes

A 3'-untranslated and C-terminal portion probe complementary to rat 5-HTT cDNA sequences 1717-2201 (Genbank accession No. M79450) was used (Hansson et al., 1998; Hoffman et al., 1998). DNA template was generated by PCR from transporter specific cDNA using T7 RNA promoter and a downstream gene-specific sequence (anti-sense). Polymerase chain reaction using 1μl rat 5-HTT cDNA, 2μl primers, 10mM

dNTPs, 25mM $MgCl_2$ and 2.5 units Taq polymerase were amplified at 95°C 1 min, 55°C for 2 min and 72°C for 2.5 min for 35 cycles with a final extension at 72°C for 7 min. DNA templates were purified from agarose gels (**Figure 10**) using Fermentas DNA Extraction Kit (Fermentas Inc., Hanover, MD). Complementary RNA probes were transcribed from gel-purified DNA template using [^{35}S]-UTP and T7 polymerase to generate anti-sense probe.

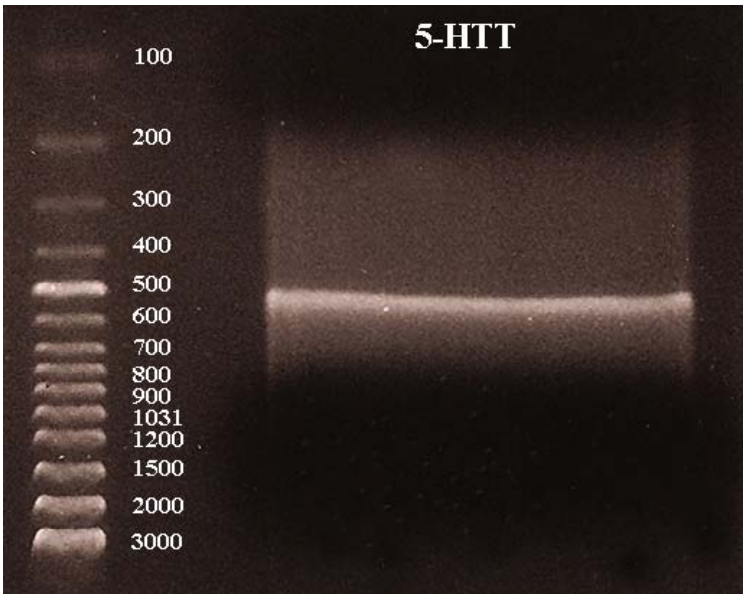


FIGURE 10. Agarose gel electrophoresis of 5-HTT DNA

4.4.1.3. Hybridization protocol and quantitative analysis

Tissue sections were fixed in 4% buffered formaldehyde for 10 min followed by 5 min incubations in phosphate-buffered-saline (0.1M PBS pH 7.40). Sections were then acetylated (0.1M triethanolamine and 0.25% acetic anhidride), delipidated, dehydrated through a graded series of ethanol and air-dried prior to hybridization. Sections were hybridized (overnight, 20-24 h, 55°C) with [^{35}S]-labeled riboprobe per 80 µl hybridization buffer consisting of 1M Tris-HCl (pH 7.4), 0.5M ethylenediaminetetraacetic acid (EDTA) (pH8.0), 5M NaCl, 100% formamide, 50% dextran-sulphate, 100x Denhardt's solution and RNAse free water. Following incubation, slides were rinsed 4x in standard saline citrate (SSC). Non-hybridized

probes were digested by incubation with RNAse A (Sigma 20 μg/ml) for 30 min. After a final wash in 0.1xSSC sections were dehydrated (Young, 1990). After drying, sections were exposed to BAS-MS 2340 Imaging Plate (FujiPhotoFilm Co. Ltd., Tokyo, Japan) for 6-13 days, depending on the intensity of the hybridization signal and scanned in a FLA-3000 phosphoimager with 16bit/pixel and 50 μm resolution, converted to "TIFF" file format by Aida Image Analyzer 3.10 software. The hybridization signals were quantified with ImageJ (NIH) imaging software.

Quantitative analysis of 5-HTT mRNA expression was carried out on coronal sections of the brains, averaging 2– 2 sections for the RPa (between -13.80 and -11.60 mm from the level of the bregma) and ROb (between -13.80 and -11.60 mm from the level of the bregma) per animal. In RPa and ROb the number of cells and number of silver grains overlying cell bodies were counted manually with light-field microscopy using an objective x40 (Olympus BX51). The average of background grain density was found less than 3 grains/cell.

For quantification we averaged (**Figure 11**) 9-9 sections for the dorsal raphe nucleus (between -9.30 and -7.30 mm from the level of the bregma), 8-8 for the median raphe nucleus (between -8.30 and -7.30 mm from the level of the bregma) and 6- 6 for the raphe magnus nucleus per animal. We have measured three rostro-caudal portions of the RMg: *rostral* (9.8–10.3 mm caudal to the level of the bregma), *middle* (10.3–10.8 mm) and *caudal* (10.8–11.3 mm).

The exact topography of these regions was determined according to a conventional rat brain atlas (Paxinos and Watson, 2005). The mean grey values over the raphe magnus nucleus, dorsal or median raphe nuclei were measured on the film autoradiography, and also over a similar size of the surrounding area that did not contain serotonergic cells. Differences measured in grey densities were used for the evaluation and statistical analysis. To obtain more precise measures for the effects of MDMA, 5-HTT expression was measured in individual cells. The sections were coated with nuclear track emulsion (NTB-3, Kodak, Rochester, NY). After a 4-week exposure at 4^{o}C, slides were developed in Kodak Dektol (Kodak, Rochester, NY), fixed and counterstained with a Giemsa stain. High magnification digital photographs were taken on the raphe magnus nucleus, dorsal and median raphe nuclei with light- and dark-field microscopy and a Qimaging, QiCAM Mono camera. For each experimental animal, 4-4

sections were analysed, and the number of silver grains were measured over randomly chosen serotonergic cell bodies (25 or 10 cells in the DR and MR, in each section, respectively). In the case of RMg we have measured all cell bodies.

The threshold was adjusted to background signal level, the subdivision of interest was outlined and the mean grey density was automatically calculated in respect to the surface area of outlined section. The mean grey density values reflect the mRNA expression of the cell.

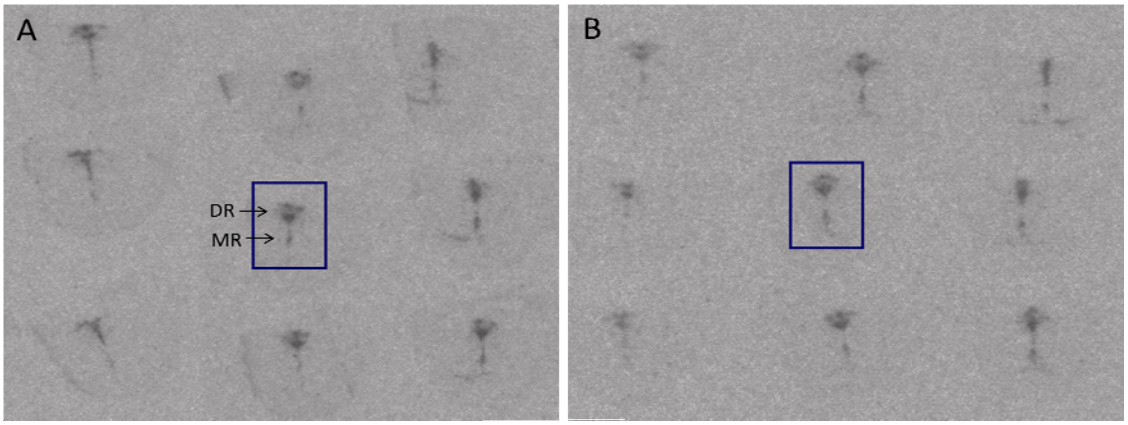


FIGURE 11. Coronal sections with radiolabeled antisense probe 21 days after a single dose of MDMA (B) and in a control group (A)
Abbreviations: DR- dorsal raphe nucleus, MR- median raphe nucleus

4.4.2. Immunohistochemistry

The animals were deeply anesthetized with nembutal, thoracotomized, and perfused transcardially of Zamboni fixative solution [4% paraformaldehyde (w/v%) and 15% saturated picric acid (v/v%) in phosphate buffer (0.1M PB pH 7.40)] 7, 21 and 180 days after the single-dose of MDMA treatments. Brains were removed and postfixed overnight at 4 ^{o}C in the fixed solution. After fixation, brains were stored in 20% glucose solution one day before sectioning. Free-floating 40 μm-thick coronal sections were cut using a freezing microtome, then the sections were stored in a cryoprotectant solution (30% ethylene glycol, 20% glycerine in 0.1 M PB) at -20 ^{o}C until the immunohistochemical procedures.

For 5-HTT immunostaining, peroxidase/DAB kit (EnVision ^{TM}, DAKO, Glostrup, Denmark) was used. The sections were brought to room temperature and washed 3x in 0.1M TBST (0.05M Tris, 0.3M NaCl containing 0.1% Tween20, pH 7.2-7.6). Endogenous peroxidase activity was blocked by application of TBST containing 0.03% H_2O_2. After rinsing with 3x TBST the slides were blocked in 1% bovine serum albumine (BSA) through 1 hour. The presence of 5-HTT was detected using a 1:3000 dilution of a rabbit polyclonal anti-5-HTT antibody (Oncogene, SanDiego, CA). The primary antiserum was diluted in antibody diluent (DAKO, Glostrup, Denmark). Sections were incubated 4 hours at room temperature. After rinsing in TBST, peroxidase labelled polymer was used for one hour. After final rinse in TBST, immune complexes were visualized in 3,3'-diaminobenzidine (DAB substrate-chromogen solution). After washing in TBST, all sections were mounted on gelatine-coated slides, air dried overnight and coverslipped with Depex.

4.4.2.1. Quantitative analysis

Monochrome digital images were made using similar light microscopic settings (Olympus BX51, objective, aperture, exposure time) at magnification of x40. Four to six representative, non-overlapping photographs were taken in each anatomical region (Paxinos and Watson, 2005): cerebral cortex layer I-II [somatosensory (SSCX), somatomotor (SMC) (3.30 - 2.30 mm caudal to the bregma) and parietal cortex (3.90 – 2.10 mm)], hippocampus (dentate gyrus (GD), CA1, CA2, CA3) (3.60 - 3.20 mm), hypothalamus [posteroventral preoptic areas (VLPO) (0.30 - 0.40 mm), lateral hypothalamic area (LH) (3.60 - 3.20 mm), posterior hypothalamic nucleus (PH) (3.60 - 4.30 mm), paraventricular nucleus (PVN) (1.50 - 2.10 mm), suprachiasmatic nucleus (SCN) (0.90 – 1.40 mm), tuberomamillary nucleus (TMN) (3.80 - 4.00 mm)], periaqueductal grey [ventrolateral part (PAGvl) (7.80 - 8.30 mm), dorsomedial part (PAGdm)], peripeduncular tegmental nucleus (PPTG) (7.80 - 8.30 mm), medial pontine reticular formation (mPRF) (8.80 - 7.80 mm), substantia nigra (5.80 – 4.80 mm), limbic cortical areas [cingulate (1.20 - +0.30 mm) and piriform cortex (3.00 – 1.80 mm)], striatum [caudate-putamen (1.80 – 0.30 mm), globus pallidus (1.80 – 0.30 mm), dorsomedial striatum (1.80 – 0.30 mm) and ventrolateral striatum (1.80 – 0.30 mm)],

bed nucleus of stria terminalis (0.30 - +0.30 mm), amygdala [central (3.30 – 1.80 mm) and medial (3.30 – 1.80 mm)], mediodorsal thalamic nucleus (2.80 – 2.30 mm), arcuate nucleus (3.80 – 2.30 mm), anterior hypothalamic area (1.80 – 1.30 mm), medial preoptic area (0.80 – 0.30 mm), lateral septal nucleus (0.30 - +1.20 mm).

To measure the density of serotonergic fibres, raw images were processed using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA; identical contrast, sharpness adjustment on each image). Quantitative analysis of serotonergic fibre density was performed using the image software analySIS (Soft Imaging System GmbH., Lakewood, CO). The positively stained fibres were distinguished from background by means of density thresholding, and the area occupied by immunostained fibre has been determined and recorded in percentage of the total image area.

4.4.3. Resident-intruder test

The resident-intruder aggression test was conducted by introducing a small (intruder) rat into the cage of a larger (resident) rat (Muehlenkamp et al., 1995). The behaviour of the animals was recorded on videotape for 15 minutes immediately after the introduction of the smaller animal into the cage (Holmes et al., 2002). The record was scored manually at a later time using a computer program designed for the purpose, as described by us previously (Kantor et al., 2000). The data were calculated from the raw scores made by a trained observer and these were included in the results.

The following behavioural elements were scored:

Biting, boxing, kicking were scored and classified as impulsive aggressive behaviours, and the incidence of each parameter, as well as their sum were calculated. The latency of each behaviour (time elapsed until the first event) was also recorded. An additional parameter, namely the latency of first impulsive aggressive behaviour (biting, boxing, and kicking) was also recorded. This was defined as the time elapsed until the appearance of the first element irrespective of whether this constituted biting or boxing or kicking. Duration and latency of ***wrestling*** were also scored and calculated. Within this parameter ***pushing*** and ***dominant posture*** were also included.

Duration of ***anogenital sniffing, grooming*** of the intruder, ***over-crawling*** and ***under-crossing*** were included and summarized within the social behaviours. From these behaviours, ***grooming the intruder*** was also analysed separately, scored and calculated. The ***motor activity*** of each resident animal was observed and scored in each of the three 5-minute periods and a single numerical score was assigned to each rat, for each period. Scoring was based on a five point scale (0-4); 0 indicating lack of movement, and 4 indicating continuous motor activity. The three scores from each of the periods were summed, yielding a score in the range 0 to 12 for each resident animal. ***Exploratory behaviour*** was scored in a similar way to locomotor activity, using a five point (0-4) scale. Scoring recorded the frequency of the following events: ***rearing, sniffing, exploration of the home cage***.

4.4.4. Vigilance studies

4.4.4.1. Surgery

Animals were chronically equipped with EEG and EMG (electromyogram) electrodes, as described previously (Filakovszky et al., 2001; Kantor et al., 2002). Briefly, stainless steel screw electrodes were implanted epidurally over the left frontal (L: 2.0 mm and A: 2.0 mm to bregma) and the left parietal cortex (L: 2.0 mm and A: 2.0 mm to lambda) for fronto-parietal EEG recordings. The ground electrode was placed over the cerebellum. In addition EMG electrodes (stainless steel spring electrodes embedded in silicon rubber, Plastics One Inc., Roanoke, VA) were placed in the muscles of the neck. Surgery was performed under halothane (2%) anaesthesia (Fluotec 3, Cyprane Limited, Keighley Yorkshire, UK) using a Kopf stereotaxic device. After a 10-day recovery period, the rats were attached to the polygraph by a flexible recording cable and an electric swivel, fixed above the cages, permitting free movement of the animals. To habituate the animals to the recording conditions, the rats were attached to the polygraph and received i.p. injections of physiological saline daily for 5 days before the experiments. The animals remained connected to the recording cables throughout the experiment.

4.4.4.2. Sleep recording and scoring

EEG, EMG and motor activity were recorded for 24 h periods, starting at light onset as described earlier (Gottesmann, 1992; Kantor et al., 2004; Kantor et al., 2005). The vigilance states were scored visually for 4 sec periods for first 2 hours after light onset using conventional criteria (Kantor et al., 2004; Kantor et al., 2005). The polygraphic recordings were classified by sleep analysis software for the 24 h recordings (SleepSign for Animal; Kissei Comtec America Inc.). Results of manual and automatic analysis showed strong correlations for all vigilance states (Kantor et al., 2004). EEG power spectra were computed for consecutive 4 sec epochs on the frequency range of 0.25 to 30 Hz (fast Fourier transformation routine, Hanning window; frequency resolution, 0.25 Hz (Kantor et al., 2005).

The sleep variables were divided into four groups: ***REM*** and ***NREM*** [light slow wave sleep (SWS-1) plus deep slow wave sleep (SWS-2)] sleep indexes, ***wake parameters*** before and at around activity (dark) onset and the ***sleep continuity***. The REM sleep parameters included REM latency [the time elapsed from sleep onset until the first 7 consecutive epochs of REM sleep (Mendelson, 1996)], duration of REM in the first hour (calculated from the beginning of light onset), time of REM maximum (the hour in which the most REM sleep occured) and the sum of REM in the light period, the passive phase (sum of duration of all REM sleep periods during 0-12 h). NREM sleep indexes included NREM latency (the time elapsed between light onset and the first consecutive NREM sleep episode lasting at least 3 min, and not interrupted by more than 14 consecutive 4 sec epochs, or a cumulated total of 240 sec not scored as NREM sleep (Deboer et al., 2003; Huber et al., 1998). NREM sleep in the first and second hour (duration of NREM phase were calculated from light onset), SWS-1 and SWS-2 latencies (the time between light onset and the first cumulated total of minimum 28 seconds of SWS-1 or SWS-2), sum of SWS-1 and SWS-2 in the light period (duration of all SWS-1 and SWS-2 sleep periods during 0-12 h). Quantitative analysis of NREM sleep, namely EEG power spectra of SWS-1 and SWS-2 were also performed as described above (Kantor et al., 2004).

The wake parameters around activity onset were the activity onset (defined as the hour when the duration of active wake (AW) increased by a minimum 50%

compared to the average in the passive phase) and the passive wake (PW) before activity onset (duration of passive wake during the hour preceding the activity onset). The sleep continuity was defined as the sum of the number of awakenings (either AW or PW) that disconnected any sleep periods [SWS-1, SWS-2, intermediate stage of sleep (IS), or REM] during light period, the passive phase (0-12 h).

4.4.5. Statistical methods

For statistical analysis, STATISTICA 7.0 (Statsoft Inc., Tulsa, OK) and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) softwares were used.

Tests for homogeneity of variances in the case of the resident- intruder test indicated that variances for duration of wrestling, social behaviour and grooming were not homogenous and thus nonparametric test were performed for these parameters. Thus results for numbers and latencies of events, motor activity and exploratory behaviour and duration of all behaviours were analysed using Kruskal-Wallis nonparametric test. Where statistical differences were detected, further comparisons were performed by Mann-Whitney U-tests. For the analysis of pretreatment-treatment interactions two-way analysis of variance (ANOVA) was used for all parameters.

Data from the 5-HTT immunohistochemistry, density of 5-HTT mRNA expressions were analyzed using two-way (treatment and time) analysis of variance and Newman-Keuls test for *post-hoc* comparisons. The REM, NREM, SWS-1 and SWS-2 latencies, the sum of REM, SWS-1 and SWS-2 in the light period, passive wake before activity onset and the fragmentation of sleep were analyzed using the same method. REM and NREM in the first and second hour were evaluated also by multivariate analysis of variance (MANOVA) for repeated measures. In case of time of REM maximum and activity onset Mann-Whitney U test was performed. Log-transformed values were used for the statistical analysis of the EEG power spectra data.

5. RESULTS

5.1. In situ hybridization

5.1.1. 5-HTT mRNA expression in the dorsal raphe nucleus

The changes in 5-HTT mRNA expression densities were quantified by film autoradiography at each timepoint studied (3, 7, 21 and 180 days after MDMA treatment) (**Figure 12A and 12B**). Alterations in 5-HTT mRNA expressions were further analysed on slides dipped into autoradiographic emulsion (**Figure 13A**). Autoradiographs illustrating the levels of 5-HTT mRNA in the DR between groups are shown in **Figure 12A**. In general, significant treatment x time interaction ($F_{3, 40}$= 6.369, p= 0.001) was found in the DR. Three days after a single injection of MDMA in the DR there was no alteration in the levels of 5-HTT mRNA densities. A significant increase was observed 7 days after drug administration (+24%, p = 0.008) (**Figure 12B**).

To obtain more sensitive measures for the effects of MDMA, 5-HTT mRNA expressions of individual cells were counted 7 and 21 days after drug or saline treatment (**Figure 14**). We noted significantly elevated 5-HTT mRNA expressions 7 days (+53%, p = 0.033) (**Figure 13A, 15A and 15C**) and significantly reduced 5-HTT mRNA expressions 21 days after MDMA treatment (-22%, p= 0.025) (**Figure 13A, 15E and 15G**). MDMA did not affect levels of 5-HTT mRNA in the dorsal raphe nucleus compared to the control group 3 and 180 days after the drug exposure (**Figure 12A and 12B**).

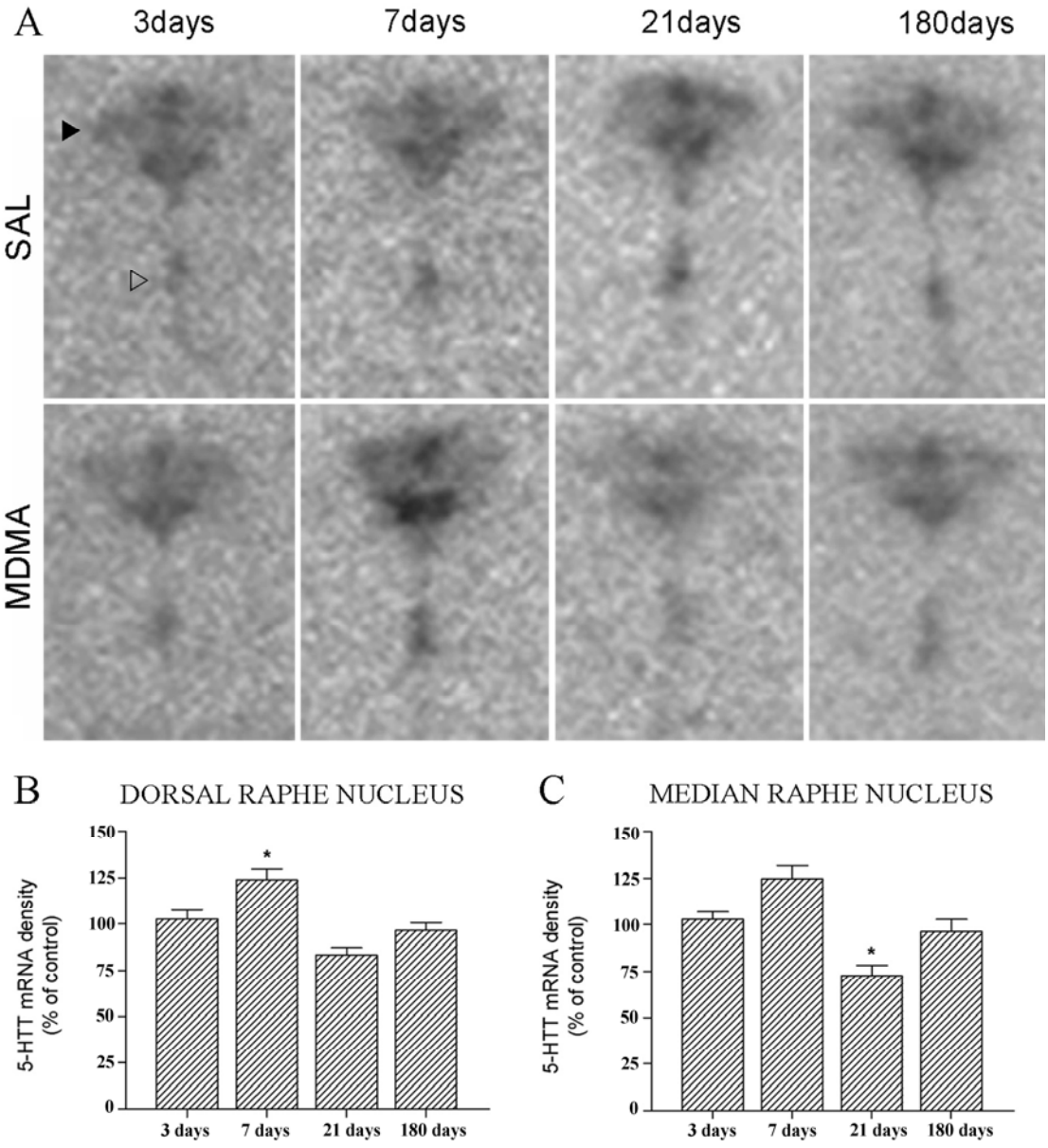


FIGURE 12. Expression of 5-HTT mRNA in control (saline) rats, and in rats at 3, 7, 21 and 180 days after treatment with a single dose of MDMA (i.p. 15 mg/kg). (A) Representative autoradiographic coronal sections through the dorsal raphe nucleus (black arrowhead, ▶) and median raphe nucleus (white arrowhead, ▷) after in-situ hybridization. Effects of MDMA on 5-HTT mRNA expression density in the dorsal raphe nucleus (B) and the median raphe nucleus (C) in rats exposed to MDMA 3, 7, 21, 90 and 180 days earlier. 5-HTT mRNA is expressed as a relative ratio compared to controls (%). Data are mean± S.E.M. (n=6). * Significantly different compared to the saline group ($p<0.05$).

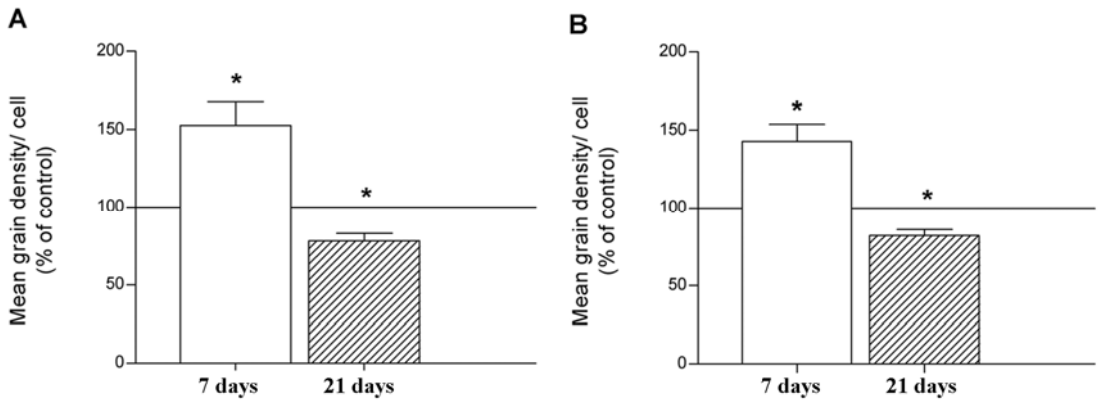


FIGURE 13. Quantification of 5-HTT mRNA expression by measuring grain densities in individual cells of the dorsal raphe (A) and median raphe (B) nuclei 7 and 21 days after MDMA treatment. Values represent mean grain density/cell ratio relative to controls. Data are mean ± SEM (n=6).

* Significantly different compared to the saline (SAL) group ($p < 0.05$).

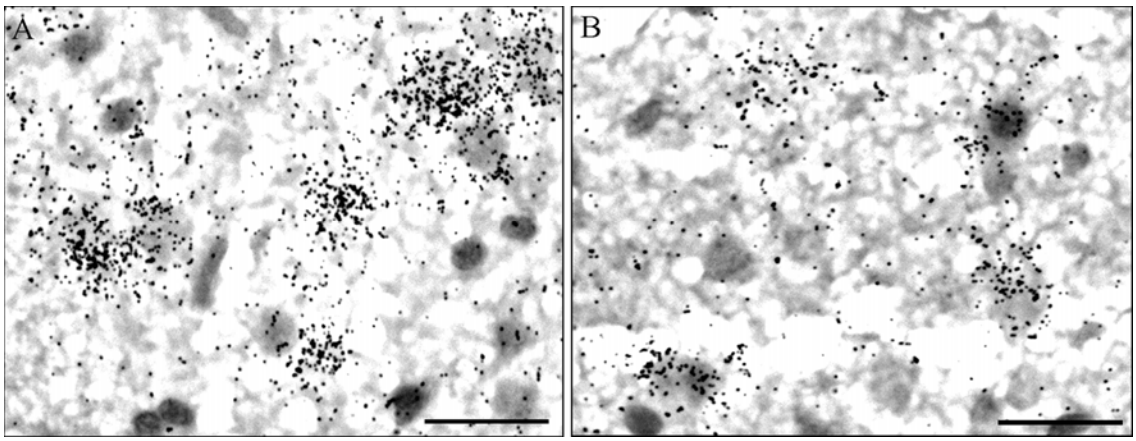


FIGURE 14. Bright-field image showing the cellular localization of 5-HTT mRNA in the dorsal raphe nucleus in control rats (A), and in rats 21 day after treatment with a single dose of MDMA (B). Panel B shows decreased grain density. Scale bars = 25μm.

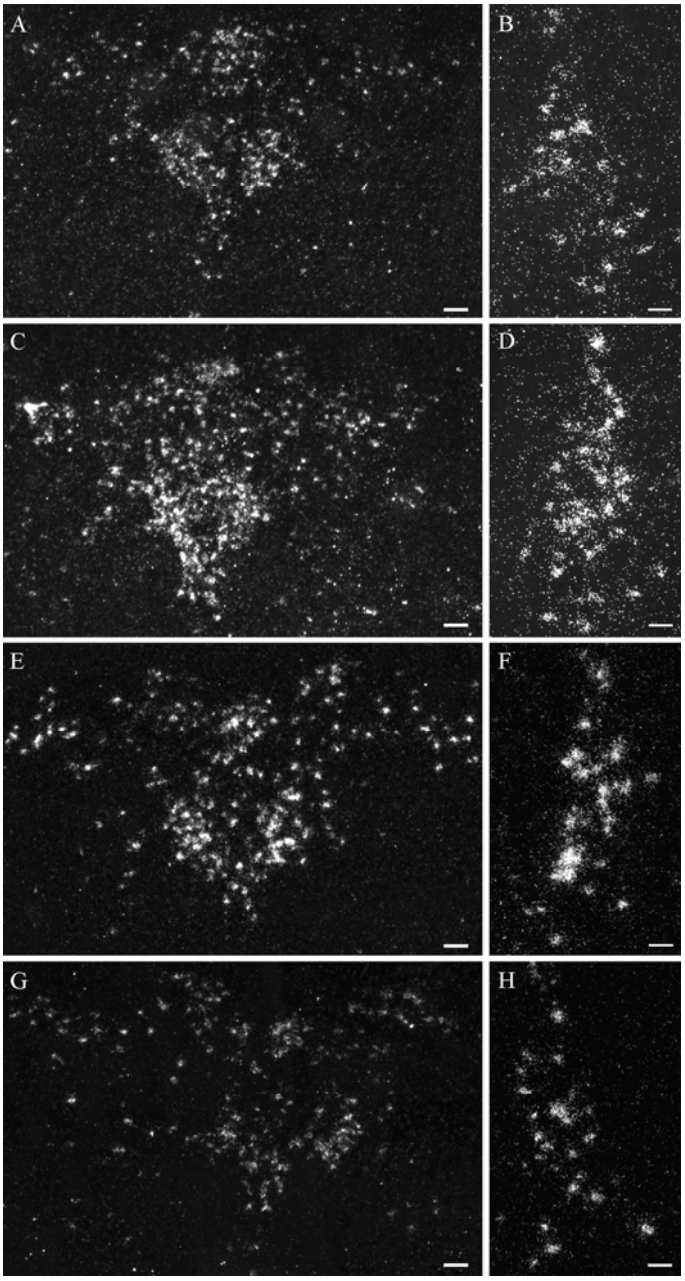


FIGURE 15. Dark-field images show 5-HTT mRNA expressions in the dorsal (A, C, E, G) and median raphe nuclei (B, D, F, H) in control (A, B, E, F) rats and in rats treated with MDMA 7 (C, D) and 21 (G, H) days earlier. 5-HTT mRNA revealed by *in situ* hybridization with [^{35}S]-labeled ribonucleotide probe. Increased 5-HTT mRNA expressions 7 days following a single dose of MDMA (C, D), as compared to controls (A, B). A marked decrease in 5-HTT mRNA is shown in panels G and H 21 days following a single dose of MDMA as compared to controls (E, F). Scale bars = 100 μm (A, C, E, G), = 50 μm (B, D, F, H).

5.1.2. 5-HTT mRNA expression in the median raphe nucleus

In the median raphe nucleus, 5-HTT mRNA densities were quantified by film autoradiography at each survival time (3, 7, 21 and 180 days after MDMA treatment) by treatment with MDMA (**Figure 12A and 12C**), and the changes in 5-HTT mRNA expressions were further compared by analysis of the signal from the slides dipped into autoradiographic emulsion, similarly to the measurements on the dorsal raphe nucleus (**Figure 13B**). In general, significant treatment x time interaction ($F_{3,39}$= 4.790, p =0.006) was found in the MR. As shown in **Figure 12C**, 5-HTT mRNA expression significantly decreased 21 days (-27%, p= 0.016) after drug exposure in the MR, but not 3 and 180 days after MDMA administration. To support these results by a more sensitive method, 5-HTT mRNA expressions of individual cells were counted 7 and 21 days after MDMA administration. We found significant increase in the levels of 5-HTT mRNA 7 days (+43%, p= 0.049) (**Figure 13B, 15B and 15D**) and significant decrease 21 days after MDMA treatment (-18%, p= 0.016) (**13B, 15F and 15H**). No differences between controls and treated animals were observed 3 and 180 days after MDMA administration (**Figure 12A and 12C**). Qualitatively, all of these effects were similar to changes observed in the DR.

5.1.3. 5-HTT mRNA expression in the nucleus raphe pallidus, obscurus and magnus

Seven days after a single dose MDMA injection elevated 5-HTT mRNA expressions were measured in the RMg (**Figure 16, 17A, B, C and D**), RPa and ROb (**Figure 19, 18A and 18B**). The average increases of 5-HTT per cell were 24% (p= 0.101) in the RMg, 35% (p= 0.003) in the RPa and 10% (p= 0.012) in the ROb (**Figure 16A, 19A and 19C**). The values of the mean grey density were different in the three different subregions of the RMg (rostral, middle and caudal). Significantly higher values were found only in the middle portion of the nucleus (46%, p= 0.019). No alterations in the levels of 5-HTT mRNA expressions or in cell numbers were detectable in raphe nuclei examined 180 days after the exposure to MDMA (**Figure 16B, 19 B and 19D**) in the investigated nuclei, although values were measured only in three saline-

treated animals. The cell density (cell numbers/nuclei) did not show any alterations in the raphe nuclei investigated 7 and 180 days after MDMA injections (**Figure 20**).

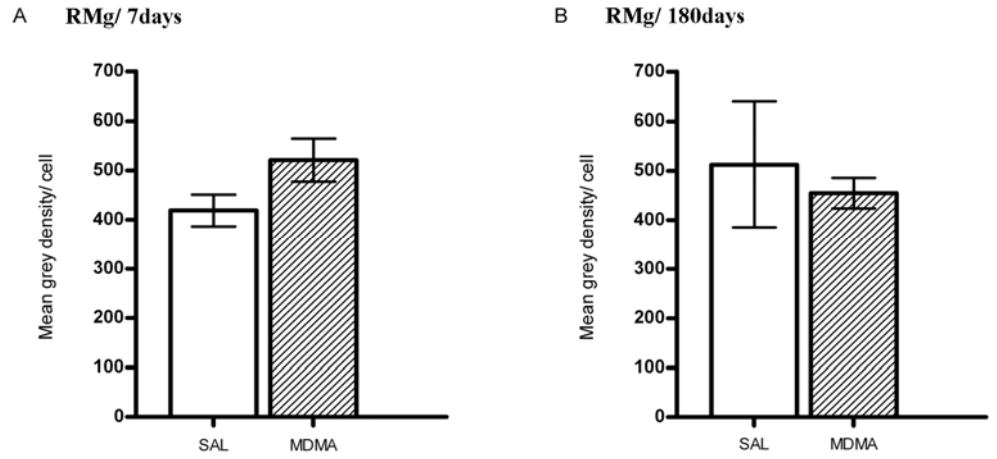


FIGURE 16. Mean grey density/cell in the raphe magnus (RMg) 7 days, (**A**) and 180 days, (**B**) after MDMA treatment.

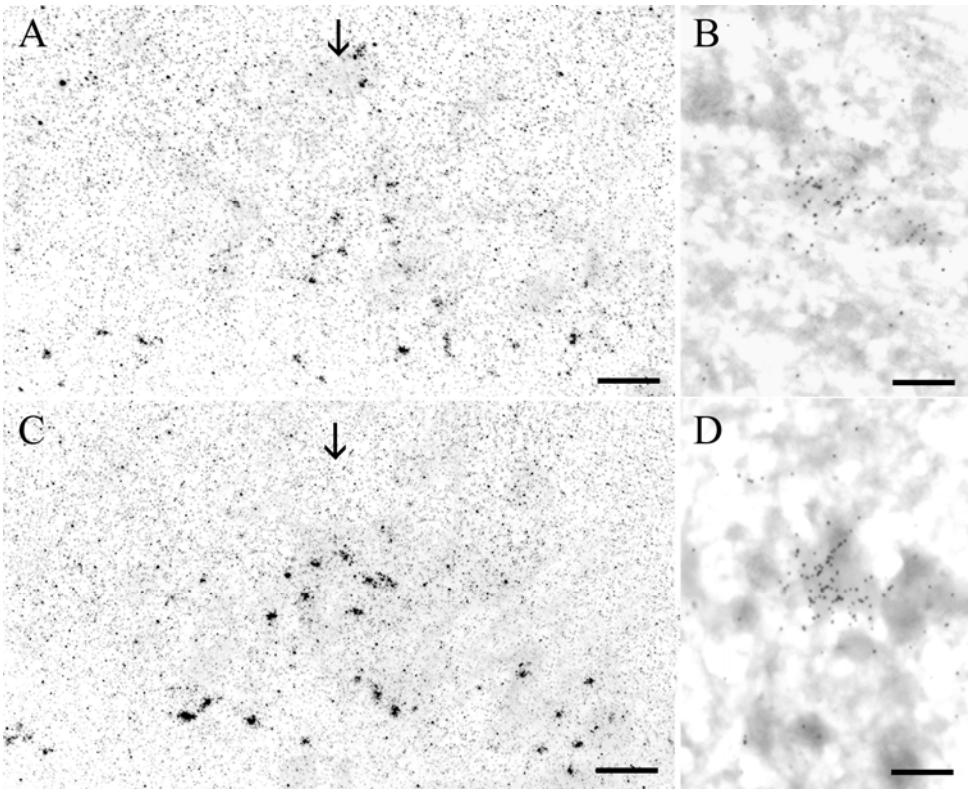


FIGURE 17. Low (**A, B**) and high (**B, D**) magnification (silver grains overlying a cell body) bright-field microphotographs show 5-HTT mRNA expressions in raphe magnus neurones 7 days after treatment with saline (**A, B**) or single dose of MDMA (**C, D**). 5-HTT mRNA is revealed by *in situ* hybridization. Arrows indicate the midline. Scale bars: **C, E**= 100 μm; **B, D**= 20 μm.

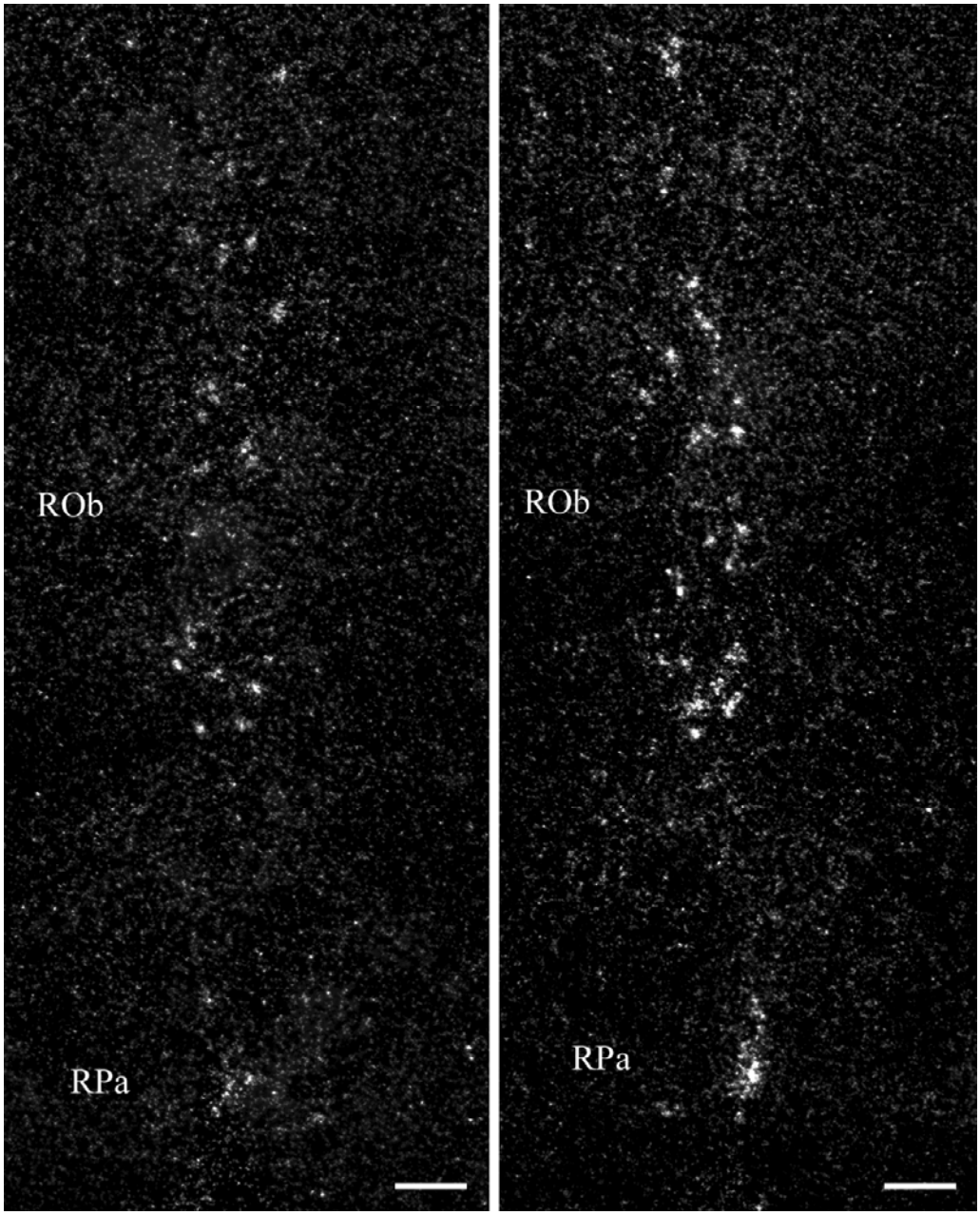


FIGURE 18. Low magnification dark-field microphotographs show mRNA expression of the serotonin transporter (5-HTT) in obscurus (ROb) and pallidus (RPa) nuclei 7 days after treatment with saline (**A**) or single dose of MDMA (**B**). 5-HTT mRNA is revealed by *in situ* hybridization. Scale bars= 100 μm.

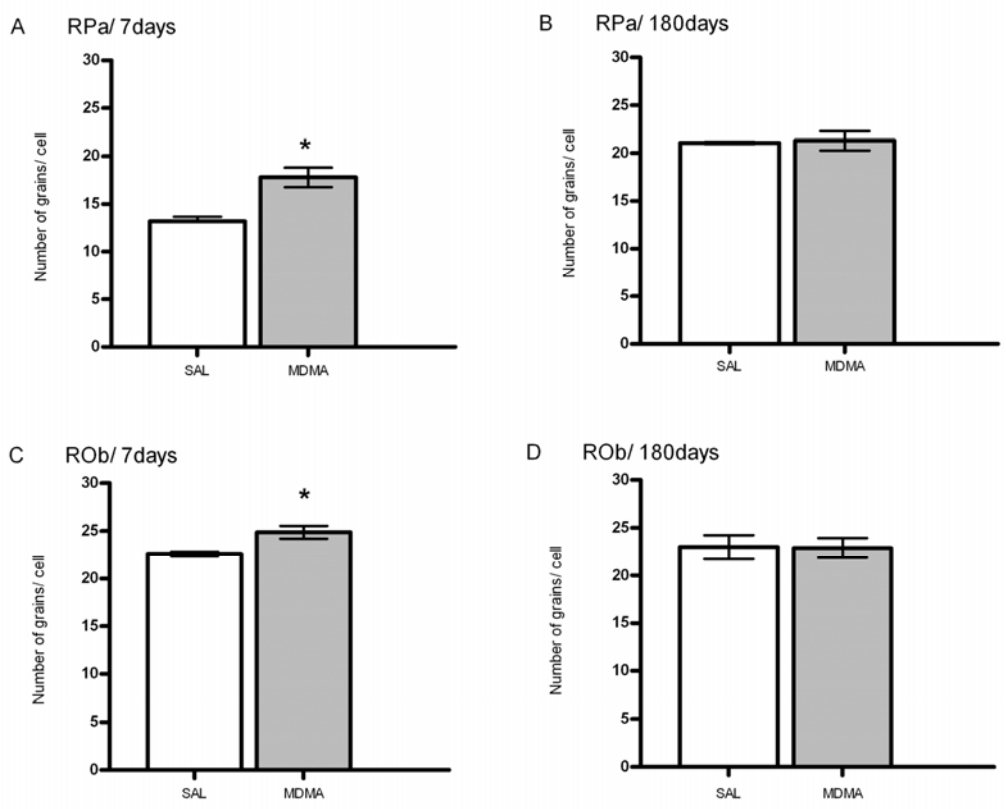


FIGURE 19. Number of grains/cell ratio in the raphe pallidus (RPa, A, B) and obscurus (ROb, C, D) 7 days [saline (SAL): $n = 6$, MDMA: $n = 7$] and 180 days (SAL $n = 3$, MDMA $n = 6$) after treatment. Data are mean ± SEM.

* Significantly different compared to the saline (SAL) group ($p < 0.05$).

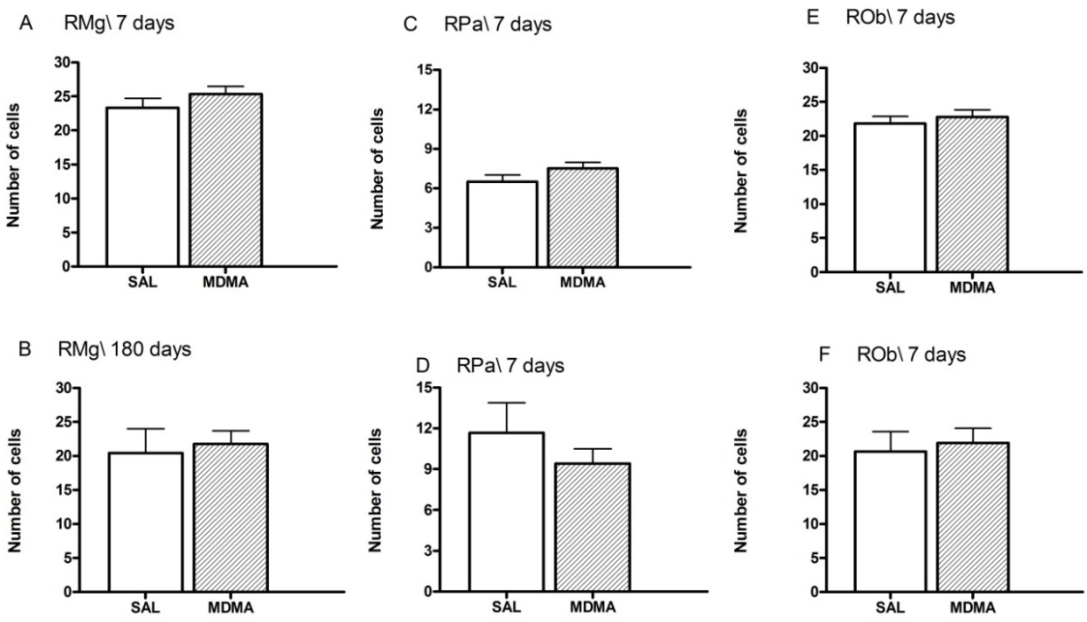


FIGURE 20. Number of cells in the raphe magnus (RMg, A, B), pallidus (RPa, C, D) and obscurus (ROb, E, F) 7 days [saline (SAL): $n = 6$, MDMA: $n = 7$] and 180 days, (**B**) (SAL $n = 3$, MDMA $n = 6$) after treatment. Data are mean ± SEM.

5.2. Density of 5-HTT immunoreactive fibres in the brain

5.2.1. Cerebral cortex and limbic cortical areas

Treatment with MDMA reduced 5-HTT-IR fibre densities both at 7 and 21 days in the somatosensory (**Figure 23**) and somatomotor cortex (**Figure 21, 22 and Table 2**). The percentage changes in the somatosensory cortex and somatomotor cortex were similar: -31%, -33%, -15%, and -36%, -32%, -14% for 7, 21 and 180 days after treatment, respectively. We found significant decrease (-35%) in the parietal cortex 21 days after drug administration. The reduction of 5-HTT-IR fibre densities was not significant 180 days after drug exposure (**Figure 22, 23**). However, the changes in both examined limbic cortical areas were significant: in the cingulate cortex -39%, -29% and in the piriform cortex -24%, -30% for 21 and 180 days after MDMA treatment, respectively.

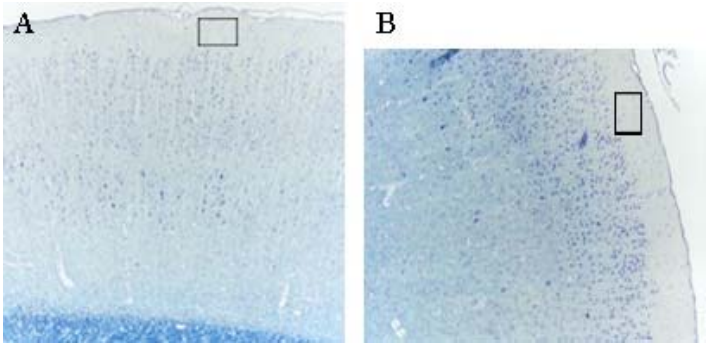


FIGURE 21. Representative photomicrographs illustrating the areas in the somatosensory (A) and somatomotor cortex (B) where the densitometry was applied (rectangles in panels A and B).

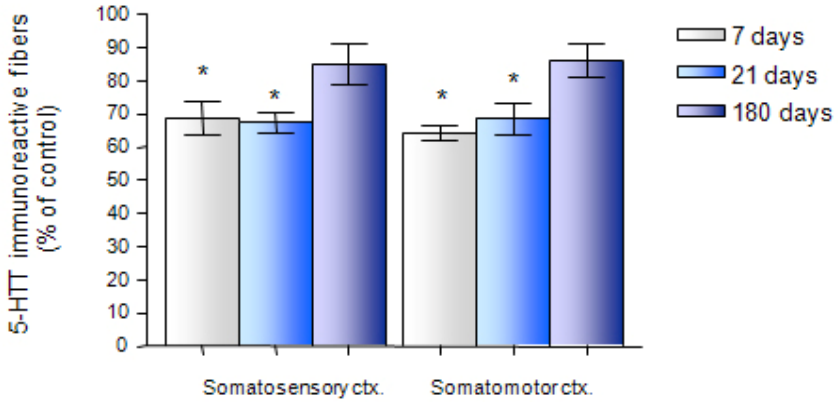


FIGURE 22. 5-HTT-IR fibre density in the somatosensory and somatomotor cortex as a relative ratio compared to controls (%). Data are mean±SEM (n=6); * Significantly different compared to the control group ($p < 0.05$).

TABLE 2.

5-HTT-IR fibre densities 7, 21 and 180 days after MDMA (15 mg/kg) treatment in brain areas of Dark Agouti rats

Brain area	7days		21days		180days	
	SAL ± SEM	MDMA ± SEM	SAL ± SEM	MDMA ± SEM	SAL ± SEM	MDMA ± SEM
Cerebral cortex						
Somatosensory cortex	6.92 ± 0.26	4.76 ± 0.35 *	6.02 ± 0.33	4.05 ± 0.18 *	6.31 ± 0.27	5.36 ± 0.40
Somatomotor cortex	7.17 ± 0.38	4.61 ± 0.16 *	6.27 ± 0.31	4.29 ± 0.28 *	6.28 ± 0.18	5.41 ± 0.32 *
Parietal cortex	nm		5.90 ± 0.38	3.83 ± 0.18 *	6.44 ± 0.31	5.97 ± 0.16
Hippocampus						
CA1	6.33 ± 0.47	4.67 ± 0.19 *	4.91 ± 0.52	3.09 ± 0.23 *	4.48 ± 0.55	2.87 ± 0.28 *
CA2	7.08 ± 0.16	4.64 ± 0.36 *	5.79 ± 0.20	3.43 ± 0.27 *	5.37 ± 0.67	3.65 ± 0.28 *
CA3	6.07 ± 0.25	4.09 ± 0.25 *	6.09 ± 0.28	3.30 ± 0.60 *	4.79 ± 0.43	3.31 ± 0.33 *
Dentate gyrus	2.78 ± 0.18	2.10 ± 0.20	2.81 ± 0.20	1.81 ± 0.11	2.80 ± 0.36	2.28 ± 0.24
Hypothalamus						
Posteroventral preoptic areas	5.88 ± 0.49	5.23 ± 0.48	5.52 ± 0.44	4.57 ± 0.19	4.91 ± 0.58	4.80 ± 0.25
Lateral hypothalamic area	6.38 ± 0.48	5.60 ± 0.43	4.96 ± 0.18	4.03 ± 0.36	5.47 ± 0.29	5.04 ± 0.32
Posterior hypothalamic nucleus	6.71 ± 0.30	5.24 ± 0.31 *	6.25 ± 0.51	3.89 ± 0.40 *	4.52 ± 0.39	3.18 ± 0.30 *
Paraventricular nucleus	5.43 ± 0.27	5.29 ± 0.46	6.08 ± 0.38	5.72 ± 0.52	5.84 ± 0.29	5.68 ± 0.27
Suprachiasmatic nucleus	6.78 ± 0.33	4.76 ± 0.34 *	5.53 ± 0.46	3.60 ± 0.19 *	4.84 ± 0.43	4.01 ± 0.28
Tuberomamillary nucleus	5.90 ± 0.19	4.78 ± 0.31 *	5.95 ± 0.36	3.86 ± 0.33 *	5.21 ± 0.37	4.02 ± 0.22 *
Brainstem						
Peripeduncular tegmental nucleus	4.70 ± 0.12	4.38 ± 0.28	4.12 ± 0.25	3.63 ± 0.33	3.93 ± 0.17	3.65 ± 0.46
Medial pontine reticular formation	4.23 ± 0.32	4.09 ± 0.35	2.93 ± 0.29	2.52 ± 0.32	3.22 ± 0.30	3.07 ± 0.38
Periaqueductal central grey						
Ventrolateral part	10.34 ± 0.29	8.82 ± 0.39	8.28 ± 0.43	5.83 ± 0.42 *	6.66 ± 0.54	5.64 ± 0.71
Dorsomedial part	9.53 ± 0.71	7.92 ± 0.58	8.74 ± 0.60	5.61 ± 0.33 *	7.73 ± 0.70	6.47 ± 0.77

Brain area	7days		21days		180days	
	SAL ± SEM	MDMA ± SEM	SAL ± SEM	MDMA ± SEM	SAL ± SEM	MDMA ± SEM
Substantia nigra	6.44 ± 0.36	5.16 ± 0.38 *	nm		7.53 ± 0.21	6.76 ± 0.47
Limbic cortical areas						
Cingulate cortex	nm		5.88 ± 0.34	3.59 ± 0.07 *	5.48 ± 0.42	4.14 ± 0.28 *
Piriform cortex	nm		8.27 ± 0.15	5.87 ± 0.36 *	8.57 ± 0.81	5.98 ± 0.57 *
Striatum and pallidum	nm					
Dorsomedial striatum	nm		4.59 ± 0.37	2.62 ± 0.18 *	5.93 ± 0.39	4.83 ± 0.25 *
Ventrolateral striatum	nm		8.23 ± 0.54	5.93 ± 0.34 *	6.88 ± 0.34	5.79 ± 0.36
Caudate-putamen	nm		7.44 ± 0.14	5.32 ± 0.26 *	6.50 ± 0.38	5.28 ± 0.39 *
Globus pallidus	nm		4.71 ± 0.43	3.03 ± 0.21 *	5.18 ± 0.33	4.15 ± 0.38
Bed nucleus of stria terminalis	nm		5.92 ± 0.26	5.16 ± 0.26	5.40 ± 0.33	4.98 ± 0.26
Amygdala	nm					
Central	nm		6.37 ± 0.36	5.51 ± 0.56	5.91 ± 0.39	5.54 ± 0.26
Medial	nm		6.86 ± 0.51	5.27 ± 0.46 *	5.63 ± 0.37	5.05 ± 0.38
Mediodorsal thalamic nucleus	nm		3.87 ± 0.42	2.91 ± 0.30	nm	
Arcuate nucleus	nm		4.57 ± 0.55	5.25 ± 0.09	nm	
Anterior hypothalamic area	nm		5.02 ± 0.56	4.70 ± 0.42	nm	
Medial preoptic area	nm		4.45 ± 0.20	4.12 ± 0.69	nm	
Lateral septal nucleus	nm		4.15 ± 0.38	3.55 ± 0.20	nm	

Data are presented as mean ± SEM of 5-6 animals per group.

* Significantly different compared to the relevant control (SAL) group ($p < 0.05$).

(nm – not measured)

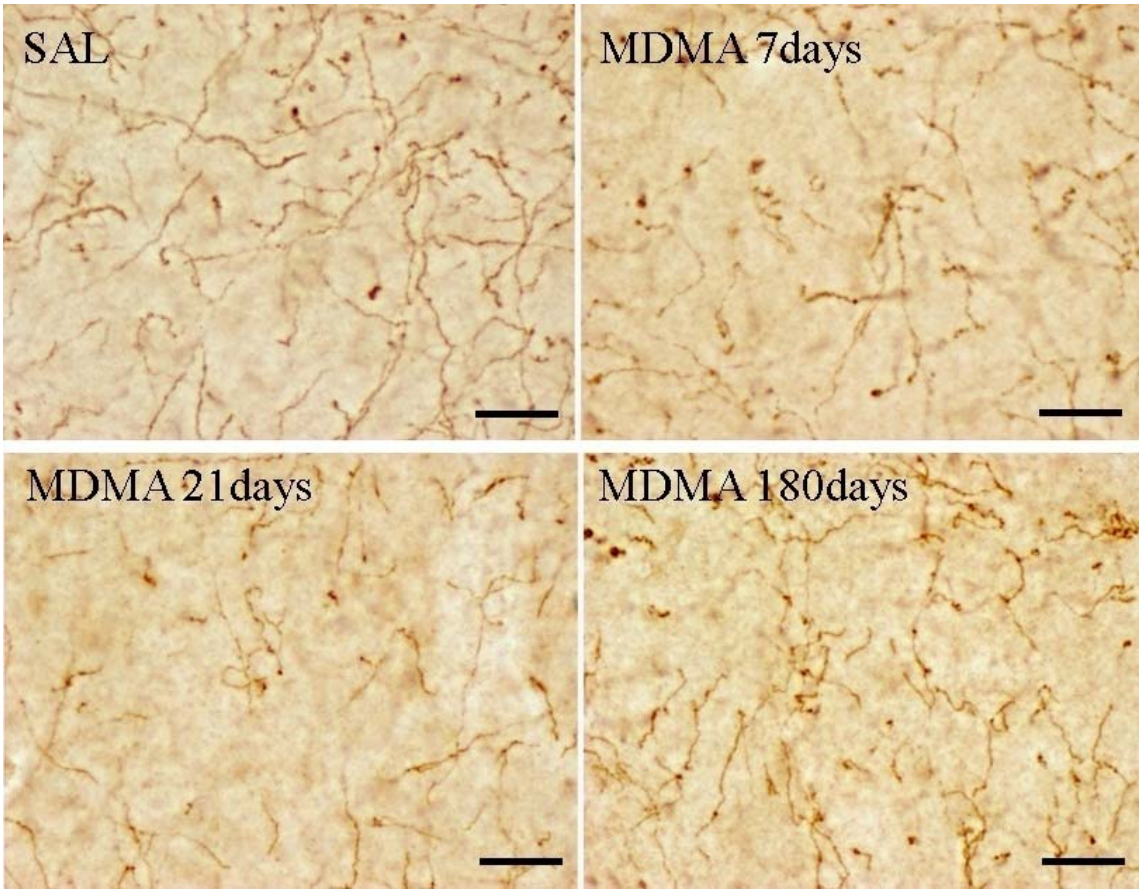


FIGURE 23. 5-HTT immunoreactive axons in the somatosensory cortex in saline (SAL) and MDMA-treated rats 7, 21 and 180 days after treatment. Scale bars = 20 μm.

5.2.2. Hippocampus

In the hippocampus, we found an overall decrease in 5-HTT-IR fibre densities, which was present from day 7 up to day 180 after the treatment (**Table 2**). We observed significant decreases in all examined hippocampal areas, except the dentate gyrus (**Figure 24, 25, 26**). Post-hoc tests indicated significantly reduced 5-HTT-IR fibre densities at all examined time points in the CA1 (-26%, -37%, -36%), CA2 (-35%, -41%, -32%) and CA3 areas (-33%, -46%, -31%) for 7, 21, 180 days after treatment, respectively. A reduction in 5-HTT-IR fibre densities was noted also in the dentate gyrus but these changes were not significant (-24%, -35%, -19% for 7, 21, 180 days after treatment, respectively).

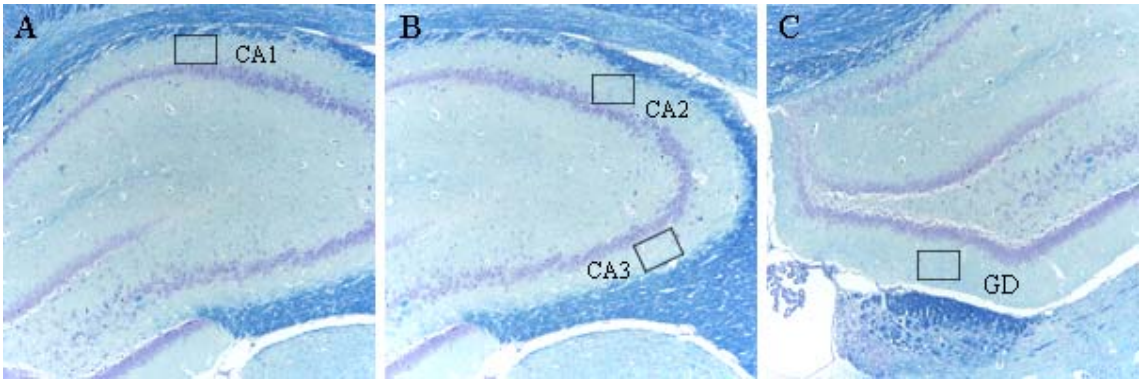


FIGURE 24. Representative photomicrographs illustrating the areas [oriens layer of CA1, CA2 and CA3; molecular layer of GD (dentate gyrus)] in the hippocampus where the densitometry was applied (rectangles in panels A - C).

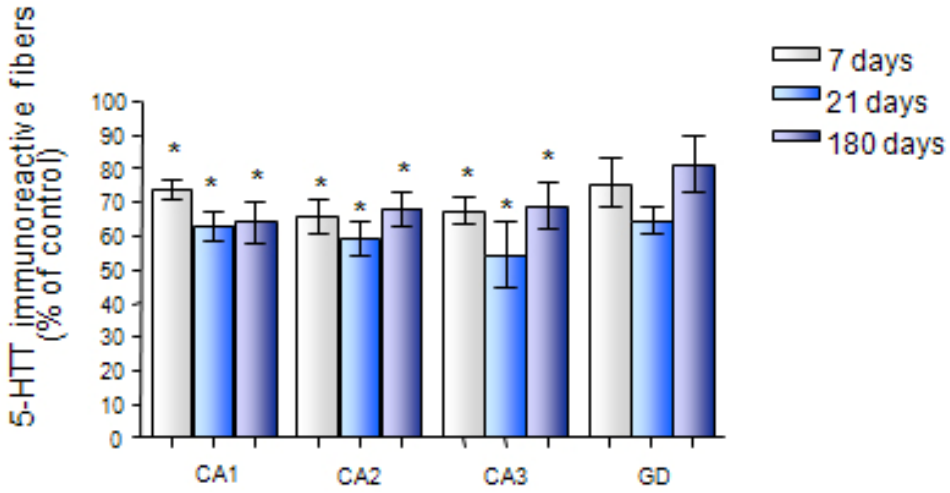


FIGURE 25. 5-HTT-IR fibre density in the hippocampus CA1, CA2, CA2 and GD (dentate gyrus) region as a relative ratio compared to controls (%).

Data are mean±SEM (n=6).

* Significantly different compared to the control group ($p < 0.05$).

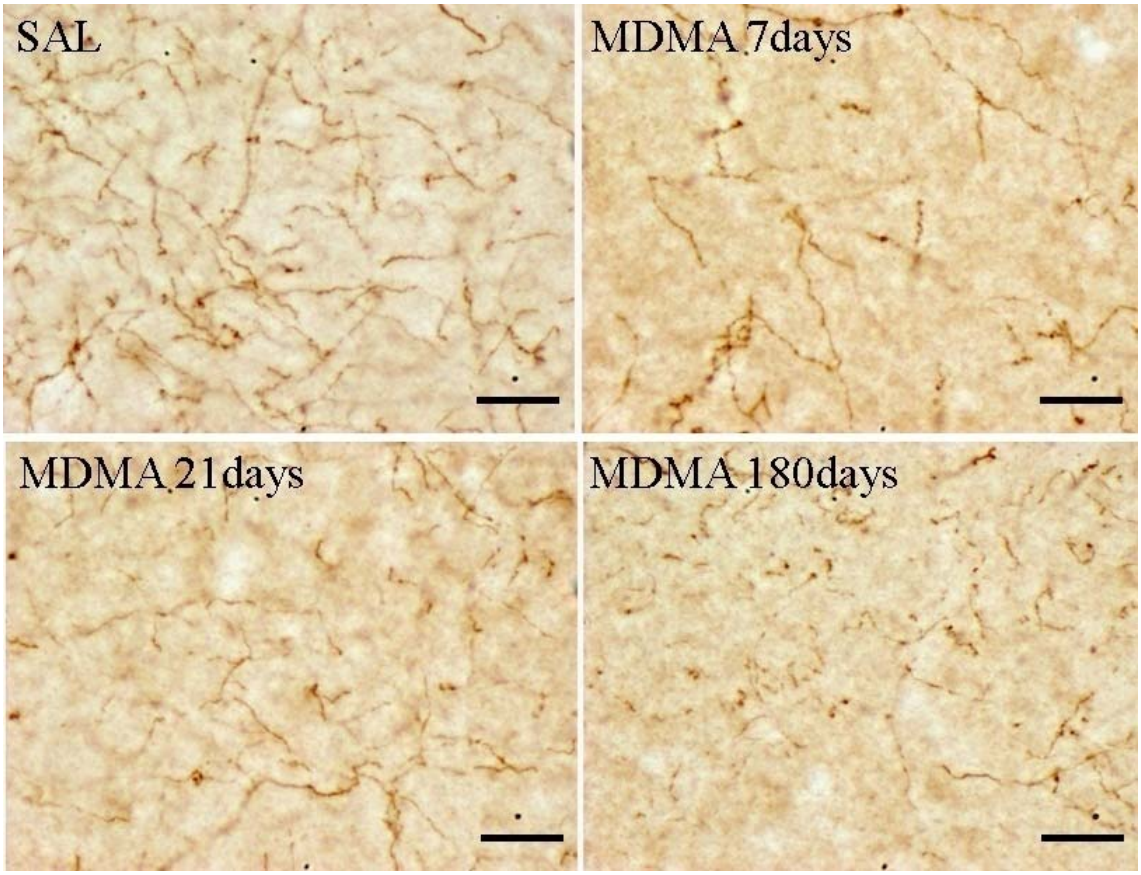


FIGURE 26. 5-HTT immunoreactive axons in the hippocampal CA1 region in saline (SAL) and MDMA-treated rats 7, 21 and 180 days after treatment. Scale bars = 20 μm.

5.2.3. Hypothalamus

The effects of MDMA on 5-HTT-IR fibre density were different in different hypothalamic nuclei and areas (**Table 2**). Reduced 5-HTT-IR fibre densities were measured in the posterior hypothalamic (PH), suprachiasmatic (SCN) and tuberomamillary (TMN) nuclei, while the lateral (LH), posteroventral preoptic areas (VLPO) and the paraventricular nucleus (PVN) were resistant to MDMA, at all time points measured (**Figure 27, 28**). Alterations in 5-HTT-IR fibre densities were the follows: LH -12%, -19%, -8%; PH -22%, -38%, -30%; SCN -30%, -35%, -17%; TMN -19%, -35%, -23%; VLPO -11%, -17%, -2%; PVN -2%, -6%, -3%, for 7, 21 and 180 days after MDMA, respectively. The changes in 5-HTT-IR fibre density were not

significant at 21 days in the hypothalamic regions which connected to the aggressive behaviour: arcuate nucleus (-14%) and anterior hypothalamic area (-7%).

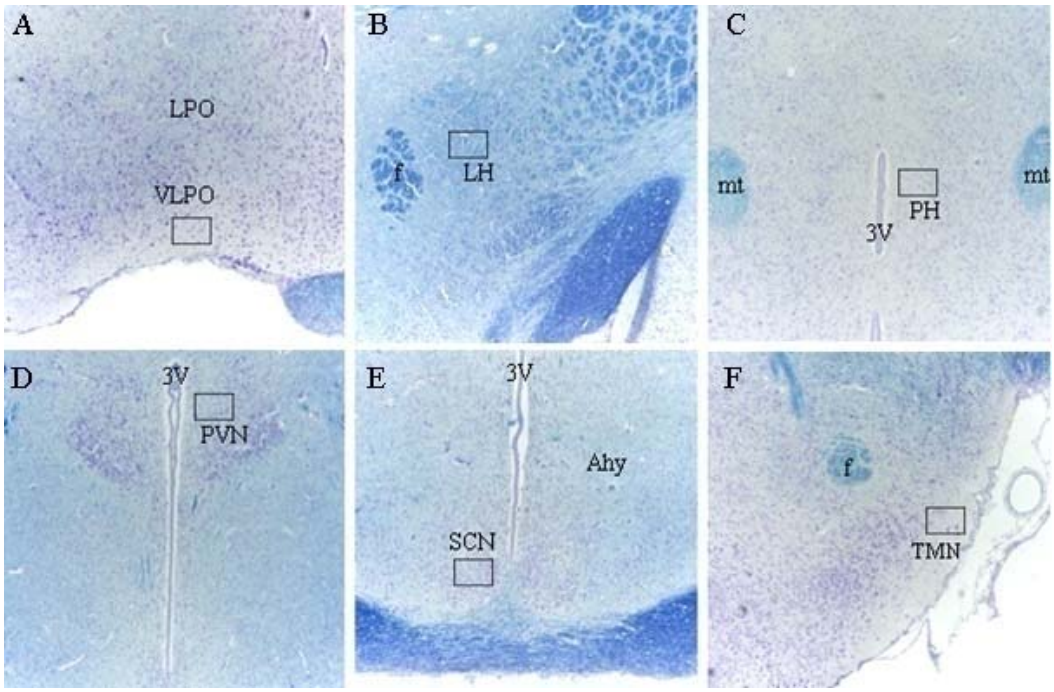


FIGURE 27. Representative photomicrographs illustrating the areas in the hypothalamus where the densitometry was applied (rectangles in panels A - F).
Abbreviations: f- fornix, mt- mammilothalamic tract, Ahy – anterior hypothalamic area, 3V – third ventricle

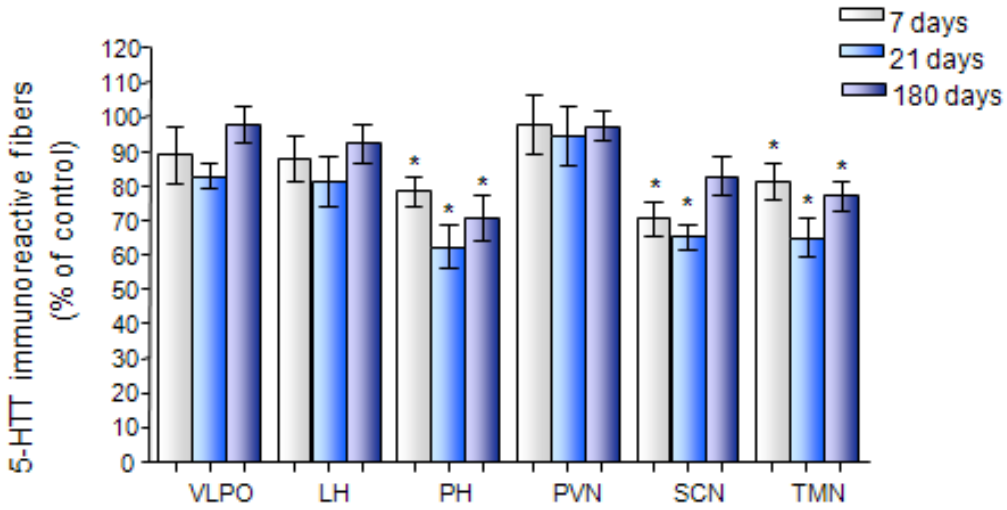


FIGURE 28. 5-HTT-IR fibre density in the different hypothalamic nuclei (VLPO, LH, PH, PVN, SCN and TMN) as a relative ratio compared to controls (%). Data are mean±SEM (n=6); * Significantly different compared to the control group ($p < 0.05$).

5.2.4. Brainstem

In general, changes in the brainstem were smaller (**Figure 29, 30**), and significant alterations were observed only in two regions, in the ventrolateral (PAGvl) and dorsomedial (PAGdm) cell groups of the periaqueductal gray matter in the midbrain, but only at 21 days (**Table 2**). Effects of MDMA on the 5-HTT-IR fibre densities in peripeduncular tegmental nucleus (PPTG) and the medial part of the pontine reticular formation (mPRF) were not significant at any time points studied. Post-hoc comparison revealed that the changes in 5-HTT-IR fibre densities were in PAGvl -15%, -30%, -15%, PAGdm -17%, -36%, -16%, PPTG -7%, -12%, -7% and mPRF -3%, -14%, -5%, for 7, 21, 180 days after treatment, respectively.

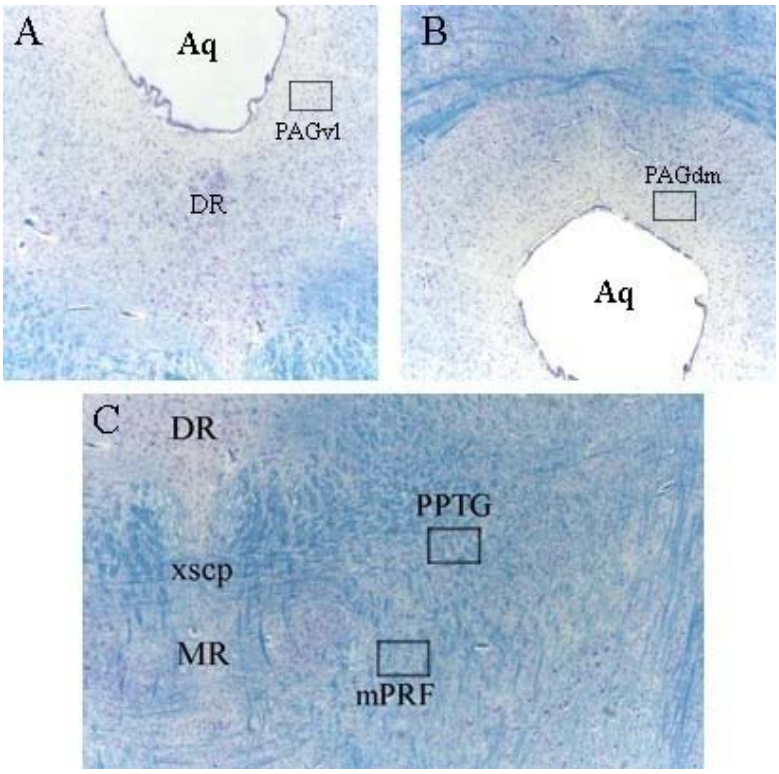


FIGURE 29.

Representative photomicrographs illustrating the areas in the brainstem where the densitometry was applied (rectangles in panels A - C).

Abbreviations: DR – dorsal raphe nucleus; xscp – decussation of superior cerebellar peduncles; MR – median raphe nucleus; Aq – aqueduct

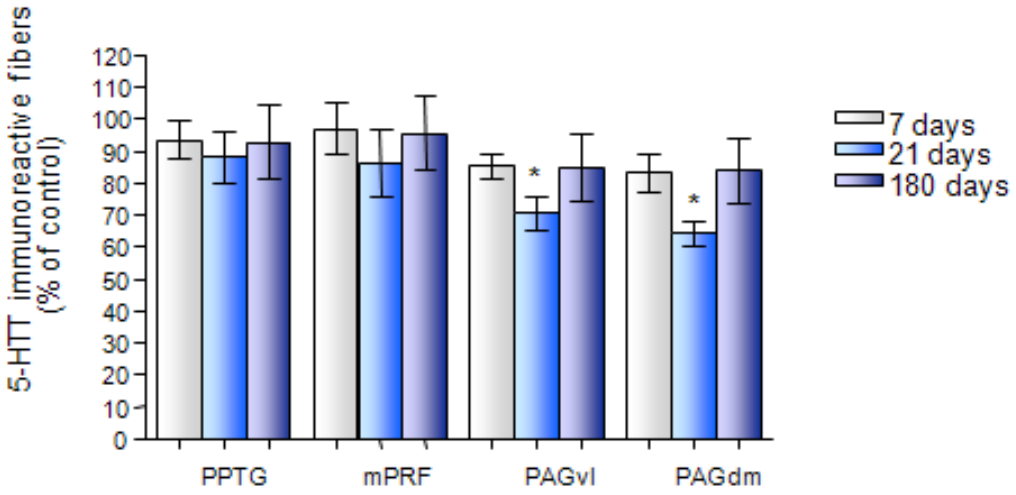


FIGURE 30. 5-HTT-IR fibre density in the brainstem as a relative ratio compared to controls (%). Data are mean±SEM (n=6); * Significantly different compared to the control group ($p < 0.05$).

5.2.5. Other investigated brain areas

In the striatum and pallidum, we observed reduced 5-HTT-IR fibre density at 21 days. However, at 180 days we found significant decrease only in the dorsomedial striatum and caudate-putamen. The percentage changes in the dorsomedial striatum, ventrolateral striatum caudate-putamen and globus pallidus were follows: -43%, -19% and -28%, -16% and -28%, -19% and -36%, -20% for 21 and 180 days after treatment, respectively (**Table 2**). Effects of MDMA on 5-HTT-IR fibre densities in the substantia nigra were significant (-20%) at 7 days (**Table 2**). In other examined brain areas (bed nucleus of stria terminalis, central amygdala, mediodorsal thalamic nucleus, medial preoptic area and lateral septal nucleus) the changes were not significant except in the medial amygdala where we observed a 23% decrease at 21 days (**Table 2**).

5.3. Resident intruder test

5.3.1. Acute effects of MDMA in drug naïve rats and in rats previously exposed to MDMA

In drug naïve rats, acute MDMA treatment caused massive decreases in all measures of aggressive behaviour, namely, incidence of biting (H=12.749, p= 0.009, **Figure 31A**), boxing (H=19.829, p< 0.001, **Figure 31B**), kicking (H=18.912, p= 0.317, **Figure 31C**), and both the sum (H=21.602, p< 0.001, **Figure 31D**) and latency of the three measures combined (H=15.832, p< 0.001, **Figure 31E**). The duration of wrestling (H=21.237, p= 0.001, **Figure 31F**) was also reduced and subsequently its latency was increased (H=20.350, p= 0.001, **Figure 31G**). Furthermore, a marked reduction was also observed in other social behaviours (H=24.340, p= 0.001, **Figure 31H**) and grooming (H=24.473, p< 0.001, **Figure 31I**) When young intruder animals were placed into the residents' home cages, both locomotor activity and exploratory behaviour of saline-pretreated resident rats was markedly increased. This was not exclusively restricted to exploration of the intruder animal, but also the home cage itself was intensively explored. Increases in locomotor activity (H=20.541, p= 0.002, **Figure 32A**) and exploratory behaviour (H=20.957, p= 0.002, **Figure 32B**) were attenuated in saline-pretreated animals after acute exposure to MDMA. In the MDMA-pretreated rats, all acute effects of MDMA were preserved (**Figure 31, 32**). The pretreatment × treatment interaction was significant only for the duration of social behaviours ($F_{1,24}$=6.394, p< 0.001) and grooming ($F_{1,24}$=14.824, p= 0.018).

5.3.2. Long-term effects of MDMA

In rats exposed to MDMA 21 days prior to the experiment there were no significant differences in the incidence of biting, boxing, the sum of biting, boxing and kicking or in the latencies of the three combined measures, and neither was there any difference in the duration of wrestling, or its latency compared to the control drug naïve group (**Figure 31**). There was, however, a significant increase in kicking (H=18.912, p= 0.010, **Figure 31C**) and a small decrease in grooming and social behaviour (H=24.473,

p= 0.008 and H=24.340, p= 0.006, for grooming and social behaviour, respectively, **Figure 31H and 31I**). Locomotor activity and exploratory behaviour were also unchanged in rats pretreated with MDMA (**Figure 32**).

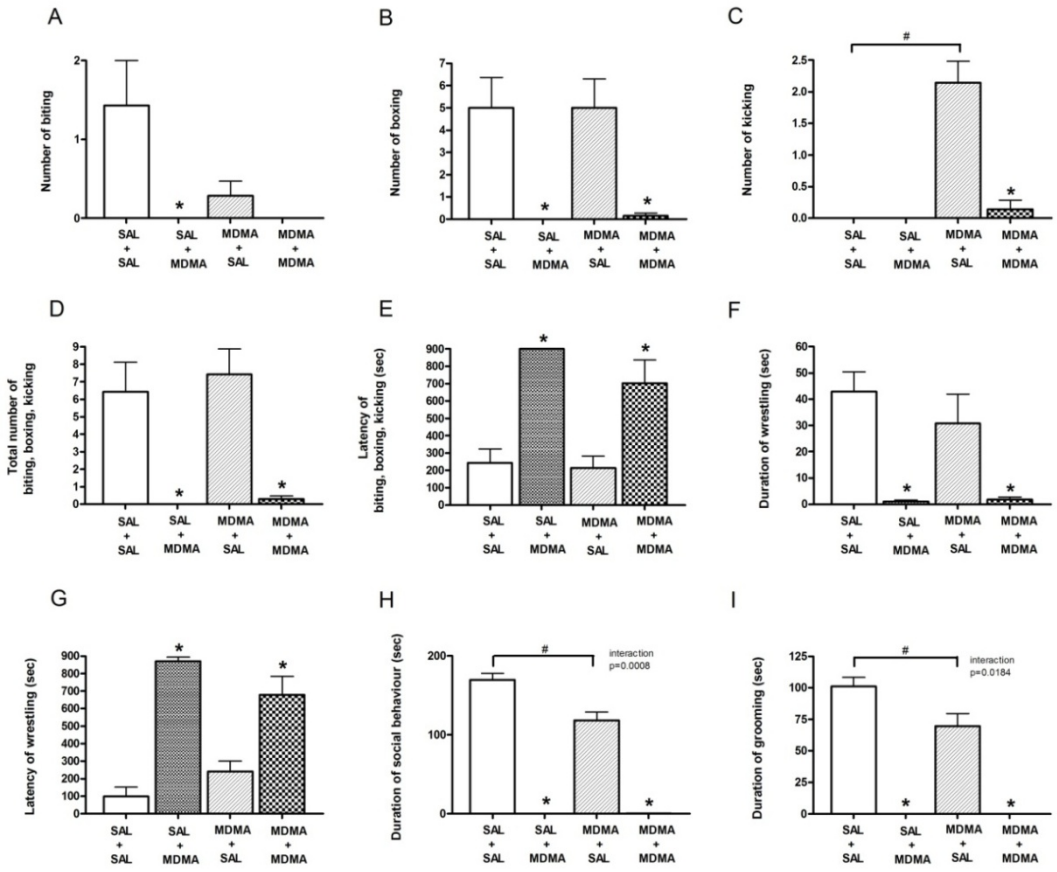


FIGURE 31. Acute effects of MDMA (15 mg/kg) upon number of biting (A), boxing (B), kicking (C), total number (D) and latency (E) of biting, boxing, kicking, duration (F) and latency (G) of wrestling, duration of social behaviour (H) and grooming (I) in drug naïve rats and in rats exposed to MDMA 21 days earlier. Each group consisted of 7 animals. Pretreatment- treatment interactions of ANOVA are significant where noted.

* Significantly different from the appropriate control with the same pre-treatment, $p<0.05$.

Significantly different from the saline-pre-treated group with the same acute treatment ($p<0.05$).

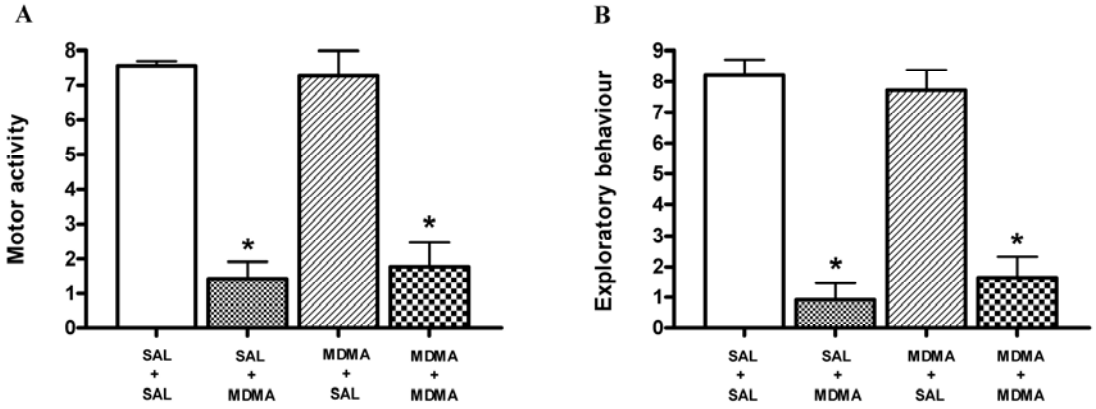


FIGURE 32. Acute effects of MDMA (15 mg/kg) on motor activity and exploratory behaviour in drug naïve rats and in rats exposed to MDMA 21 days earlier. Each group consisted of 7 animals. * Significantly different from the appropriate control with the same pre-treatment ($p<0.05$).

5.4. Effects of MDMA on sleep parameters

5.4.1. REM sleep

Complete 24 h recordings of REM sleep are shown in **Figure 33A–C**. In addition, four parameters, REM latency, REM in the first hour of passive phase, time of REM maximum and the sum of REM in the light period (passive phase) were measured and calculated for the characterization of onset and duration of REM sleep (**Table 3**). Significant decreases in REM latency were detected 7 and 21 days after MDMA administration (treatment effect $F_{1,36}= 22.880$, $p< 0.0001$). Changes in REM latency were –55%, –53%, and –29%, 7, 21 and 180 days after MDMA, respectively. In parallel, increased duration of REM (+245%) was found in the first hour of the passive phase 21 days after MDMA (treatment effect $F_{1,46}= 7.689$, $p= 0.0079$). Duration of REM sleep analyzed during the whole light period (–15%, –34%, –15% at 7, 21 and 180 days after treatment, respectively) was not significantly affected by MDMA treatment. At 7 days, some MDMA-treated animals showed an extreme time course of REM during the passive phase; REM activity showed a biphasic pattern, with the higher peak occurring very late, close to the dark phase (**Figure 33A**).

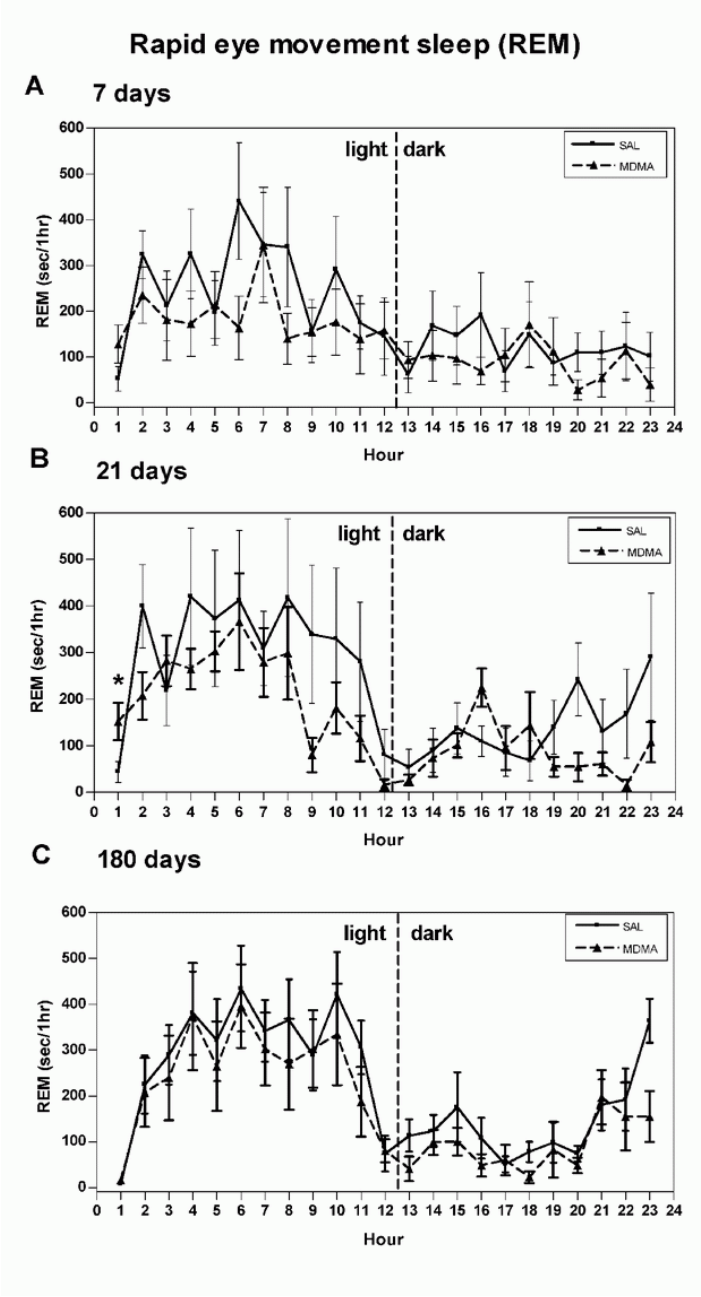


FIGURE 33. Twenty-four hour recordings of rapid eye movement (REM) sleep in MDMA and saline (SAL)-treated animals 7 (A), 21 (B) and 180 (C) days after treatment. Each point represents mean values ± SEM (n= 6-8) of the time spent in REM sleep within each hour.

* Significantly different compared to the SAL group ($p < 0.05$).

TABLE 3. Sleep parameters at 7, 21 and 180 day after MDMA (15 mg/kg) treatment

	7days		21days		180days	
Sleep parameter	**SAL ± SEM**	**MDMA ± SEM**	**SAL ± SEM**	**MDMA ± SEM**	**SAL ± SEM**	**MDMA ± SEM**
REM sleep						
REM latency (sec)	3745 ± 446	1676 ± 249*	2937 ± 364	1383 ± 160*	3511 ± 647	2485 ± 451
REM in 1st hour (sec)	53.0 ± 26.9	128.4 ± 41.1	44.0 ± 22.6	152.0 ± 40.3*	8.0 ± 5.3	15.6 ± 7.8
Time of REM max (hour)	4.88 ± 0.97	8.00 ± 1.11	4.71 ± 0.97	6.13 ± 0.58	6.88 ± 0.79	6.25 ± 0.90
Sum of REM in the light period (sec)	2485 ± 684	2107 ± 429	3742 ± 1277	2453 ± 458	3465 ± 660	2959 ± 766
NREM sleep						
NREM latency (sec)	1798 ± 367	1558 ± 269	1986 ± 412	1687 ± 515	2201 ± 394	2195 ± 429
NREM in 1st hour (sec)	1107 ± 217	1414 ± 167	891 ± 127	1410 ± 248	663 ± 144	801 ± 179
NREM in 2nd hour (sec)	2230 ± 196	1998 ± 113	2280 ± 188	2188 ± 157	2136 ± 95	2133 ± 122
SWS-1 latency (sec)	1585 ± 395	1717 ± 250	1528 ± 458	746 ± 225	2008 ± 254	2064 ± 315
SWS-1 delta power (μV^2/Hz)	3218 ± 254	4874 ± 588*	3066 ± 139	3461 ± 87	2602 ± 205	2527 ± 456
Sum of SWS-1 in the light period (sec)	7259 ± 751	6910 ± 1139	8222 ± 770	4444 ± 287*	10751 ± 1076	12473 ± 1071
SWS-2 latency (sec)	1993 ± 465	1962 ± 228	2208 ± 425	2069 ± 461	3578 ± 358	3610 ± 302
SWS-2 delta power (μV^2/Hz)	8813 ± 1230	12005 ± 1394	7255 ± 632	11478 ± 1108*	4727 ± 321	5313 ± 723
Sum of SWS-2 in the light period (sec)	16779 ± 1400	18502 ± 1523	16336 ± 996	20180 ± 591	10776 ± 872	9738 ± 1510
Wake parameters around activity onset						
Activity onset (hour)	9.38 ± 0.19	11.00 ± 0.72	9.67 ± 0.21	9.63 ± 0.42	10.38 ± 0.60	10.38 ± 0.53
Passive wake before activity onset (sec)	501.5 ± 87.5	528.6 ± 128.2	604.0 ± 82.8	435.5 ± 77.5	611.5 ± 141.1	483.5 ± 111.1
Sleep continuity						
Number of wakes in the light period	209.6 ± 20.1	384.1 ± 42.1*	228.6 ± 35.0	255.8 ± 35.8	176.4 ± 28.9	119.8 ± 13.9

Data are presented as mean±SEM of 7–9 animals per group. REM, rapid eye movement; NREM, non-rapid eye movement; SWS-1, light slow wave sleep; SWS-2, deep slow wave sleep. *Significantly different compared to the saline (SAL) group (p<0.05).

5.4.2. NREM sleep

Nine parameters (NREM latency, NREM in first and second hour, SWS-1 and SWS-2 latency, delta power and the sum of SWS-1 and SWS-2 in the light period) were measured and calculated for the characterization of onset, duration and quality of NREM sleep (**Table 3; Figure 34**). Differences in NREM latency were not significant at any time-point (–13%, –15%, 0% at 7, 21 and 180 days after treatment, respectively). Neither SWS-1 (+8%, –51%, 3% at 7, 21 and 180 days after treatment, respectively) nor SWS-2 latencies (-2%, -6%, 1% at 7, 21 and 180 days after treatment, respectively) were significantly altered by the drug. None of the parameters used for characterization of duration of NREM, NREM in the first hour (+28%, +58%, +21% at 7, 21 and 180 days after treatment, respectively) and second hour (–10%, –4%, 0% at 7, 21 and 180 days after treatment, respectively) were altered by MDMA at any time-point studied. Significant decreases in the sum of SWS-1 in the whole light period were found 21 days after MDMA treatment (treatment × time interaction $F_{2,40}$= 4.847, p= 0.013; +8%, –51%, –3% at 7, 21 and 180 days after treatment, respectively). Delta power measured either in SWS-1 or in SWS-2 was increased by MDMA at both 7 and 21 days after treatment (**Figure 34 A, B, D and E**). Changes in the lower frequencies (1 and 2 Hz) were even more consistent. Interestingly, at day 7, the increase in delta power was more pronounced in SWS-1; the increase shifted mainly to SWS-2 at 21 days (**Figure 34A and 34E**). In control rats, SWS-2 latency (time effect $F_{2,42}$= 10.815, p= 0.00016) was increased and the sum of SWS-2 (time effect $F_{2,40}$= 28.235, p<0.0001) in the light period was decreased in the old compared to the younger animals.

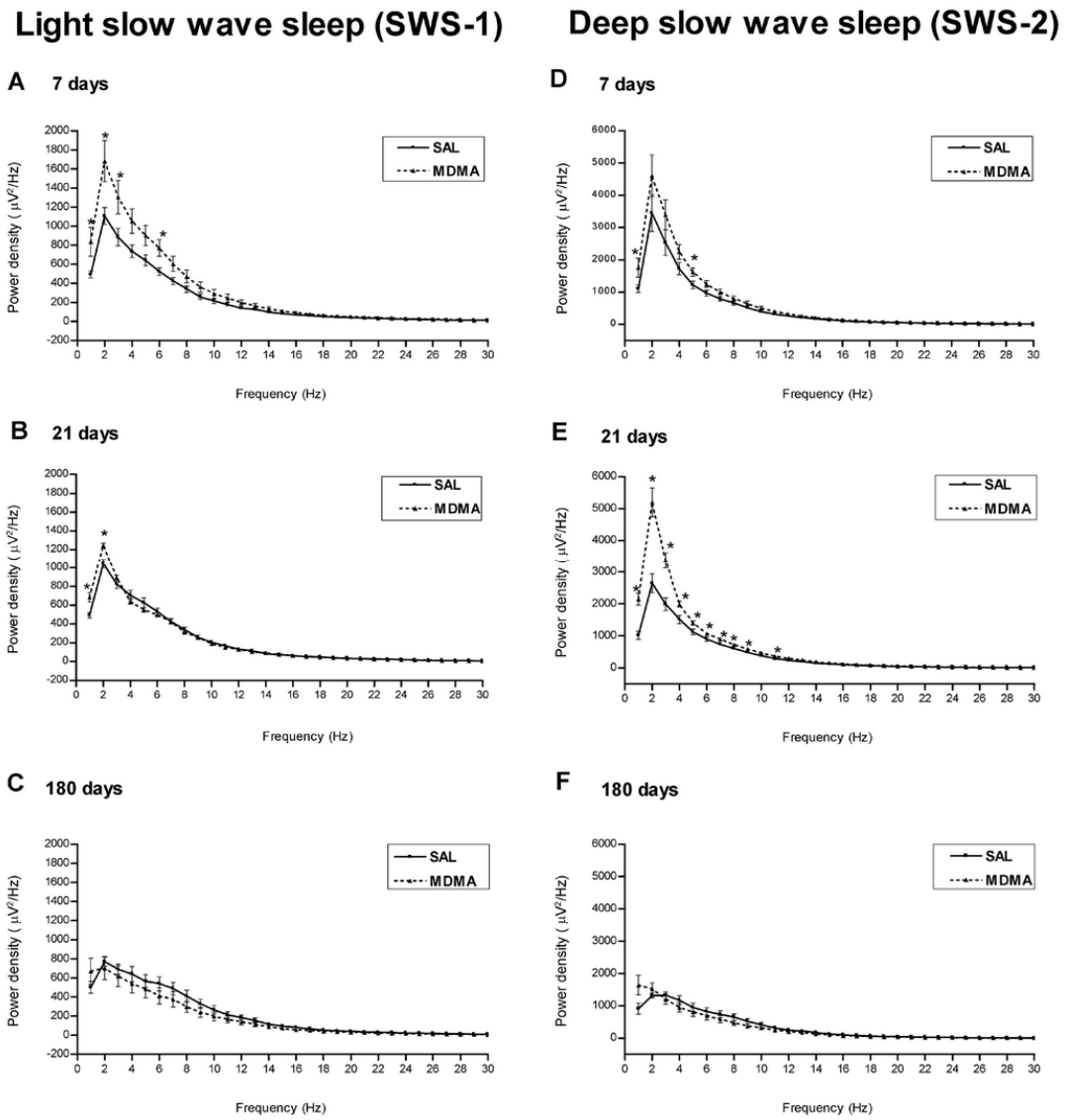


FIGURE 34. EEG power spectra (absolute power density) in slow wave sleep (SWS) -1 (A, B, C) and SWS-2 phase (D, E, F) 7 (A, D), 21 (B, E), and 180 days (C, F) after MDMA treatment. Each point represents mean values ± SEM (n= 8) of 1Hz bins in a 2 h recording period beginning at light onset.

* Significantly different compared to the saline (SAL) group ($p < 0.05$).

5.4.3. Wake parameters around activity onset

Activity onset preceding the active phase was determined for each animal, in each experimental group. Activity was increased by 50% compared to the preceding 8-h

resting period 2–3 h before lights off. No significant MDMA effect was found for this parameter (+17%, 0%, 0% at 7, 21 and 180 days after treatment, respectively). Duration of passive wake during the hour preceding the activity alteration was also calculated. Changes in the duration of passive wake before activity onset were +5%, –28%, –21% at 7, 21 and 180 days after treatment, respectively. None of these parameters were significantly altered by MDMA treatment (**Table 3**).

5.4.4. Sleep continuity

The number of wake periods during the 12 h passive phase was used to characterize sleep continuity. MDMA caused a significant increase in this parameter but only at the 7th day (+83%, +12%, –32% at 7, 21 and 180 days after treatment, respectively) (**Table 3**). The treatment × time interaction ($F_{2,41}$= 7.306, p= 0.0019) was significant.

6. DISCUSSION

Parallel measurements of 5-HTT mRNA expression in five raphe nuclei, 5-HTT-IR fibre density in several brain areas, and more than a dozen functional measures of sleep at three time-points within 180 days after MDMA administration allowed us to compare sleep disturbance with morphometric data and functions of raphe nuclei during a long period of time when the brain serotonergic system was damaged or partially recovered. Furthermore, we performed resident-intruder test to study the aggressive behaviour in drug-naïve rats and also in rats previously exposed to MDMA 21 days earlier.

In our study, MDMA was administered to Dark Agouti rats at a dose of 15 mg/kg. Although there are considerable strain differences in the susceptibility to MDMA-induced long-term serotonergic depletion, many authors suggests that a single, 15 mg/kg injection of MDMA in rats may more accurately model the possible exposure to the drug in humans, particularly when pharmacokinetic scaling is applied to body size and basal metabolic rate (Colado and Green, 1995; Malpass et al., 1999). In contrast, it is less likely that previously used models in which, typically, rats are treated with a higher dose (20 mg/kg) twice a day for four consecutive days adequately reflect human

experience. Moreover, Dark Agouti rats show decreased microsomal CYP2D6 isoenzyme activity, which plays an important role in the metabolism of MDMA. As a consequence, the Dark Agouti rat is considered to be a poor or moderate metaboliser of the drug with MDMA pharmacokinetics that is similar to those which may be found in particularly vulnerable human users (Balogh et al., 2004; O'Shea et al., 1998; Quate et al., 2004). Such users might represent a genetically-defined human sub-population in which clinical complications are more likely to occur.

Human studies are retrospective and are undermined by several factors that are impossible to control (Curran et al., 2004; Morgan, 2000; Parrott et al., 2000), furthermore, the variation in the metabolic rate that is crucial in the effect of MDMA is very high. These complications include inaccurate reporting of the doses of MDMA taken, the duration and frequency of drug use, and non-reporting of the concurrent use of other substances, licit or illicit (Green et al., 1995; Morgan, 2000). Interindividual variability of serotonergic damage measured by imaging techniques in humans is also very high (McCann et al., 1998; McCann et al., 2005), but the results of these studies are in many cases not significant. Therefore we assumed that a single dose treatment with significant axotomy may cause changes in the functional parameters. Some other data suggest that this dose in rats is in the higher dose range (O'Shea et al., 1998). Indeed, the lesion is significant in our rats; and significant decrease by brain imaging was found only in heavy ecstasy users (Ricaurte et al., 2000a). This suggests that our dose regimen is relevant to heavy ecstasy use in humans or at least it mimics some effect of heavy ecstasy use.

Under room temperature (20–22°C), MDMA administration to rats has generally been reported to produce a marked hyperthermic response of approximately +1–2°C (Broening et al., 1995; Colado et al., 1993; Dafters, 1994; Nash et al., 1988). In our other study we found that the average body temperature was 1.4°C higher in MDMA-treated rats than in saline-treated rats (Kovacs et al., 2007). In contrast, this change cannot be considered great compared to results from humans where hyperthermia was found to be one of the major symptoms of acute MDMA-induced toxicity, and body temperatures of over 43°C have been reported.

All the timepoints we selected are after the acute effect period, however, the majority of studies carried out in humans are based on weekend drug consumption

(Curran and Travill, 1997; Parrott, 2001). Effects such as fatigue, low mood, concentration and memory difficulties, reduction of appetite, etc. last for about a week or months (Morgan, 2000; Parrott, 2001). At the third week morphological studies indicate significant axon damage which has been stabilised, however, regeneration has not started.

6.1. Morphological effects of MDMA

In the present study, 5-HTT mRNA expression was measured in the two largest group of ascending projecting serotonergic neurons, the DR and the MR. A transient increase followed by a transient decrease and a complete recovery was found during the 180-day period in dorsal and median raphe nuclei. The effects of MDMA on 5-HTT mRNA expression in DR and MR did not differ in time or extent.

In the examined three descending raphe nuclei, RMg, ROb and RPa, we observed up-regulation of 5-HTT mRNA expressions at 7 days and also a complete recovery at 180 days. The up-regulation of 5-HTT mRNA expressions 7 days after MDMA treatment in all studied raphe nuclei may be explained by a compensatory phenomenon following decreased axonal transport or a possible distal down-regulation of the expression of 5-HTT (Kovacs et al., 2007).

In addition, 5-HTT-IR fibre density was also measured in a number of brain regions. Decreases were detected in several brain areas, from the brainstem up to the cerebral cortex at 7 and 21 days. Evidence for partial recovery was found in most affected brain regions by 180 days. Our earlier results show diminished TPH- fibre density in several brain regions 3 days, 1 and 3 weeks after the administration of MDMA (Adori et al., 2006; Ando et al., 2006).

It is well established for several species (Hornung et al., 1990; Kosofsky and Molliver, 1987; Mulligan and Tork, 1988) that the cerebral cortex contains at least two major types of 5-HT fibres. It was suggested that the fine fibres are derived from the DR, while thicker fibres are derived from the MR (Kosofsky and Molliver, 1987; Mulligan and Tork, 1988). It has been reported previously that the fine serotonergic fibres are selectively vulnerable to a number of amphetamine derivatives (Mamounas and Molliver, 1988; O'Hearn et al., 1988). However, more recent neuroanatomical and

biochemical studies suggest that DR selectivity of these amphetamines may not be exclusive (Haring et al., 1992; Hensler et al., 1994; Mamounas and Molliver, 1988; McQuade and Sharp, 1995; O'Hearn et al., 1988). Data obtained from these studies are in accordance with the observation that fine fibres arise from both raphe nuclei (Morin and Meyer-Bernstein, 1999). Our immunohistochemical data support the conclusion that axons of MR origin are also affected by MDMA. The origin of serotonergic projections to the SCN is almost exclusively the MR (Glass et al., 2003; Meyer-Bernstein et al., 1999), and MR fibres project heavily throughout the entire hippocampus (Azmitia and Segal, 1978; Conrad et al., 1974; Vertes and Martin, 1988). The SCN and the three CA areas of the hippocampus were strongly affected by MDMA in our experiment. Furthermore, changes in 5-HTT mRNA expression in the DR and MR were similar in the present study. These data support the view that axons of both the DR and MR origin are temporarily damaged by the drug.

The spinal cord receives a strong and highly organized 5-HT projection (Anderson et al., 1989). The major source of 5-HT innervation to the spinal cord arises from the caudal groups of 5-HT cells (Halliday et al., 1995; Hökfelt et al., 1978; Skagerberg and Bjorklund, 1985). The RMg innervates mainly the dorsal horn. The 5-HT cells in ROb and RPa project to the ventral horn and the intermediolateral column (Bacon et al., 1990; Törk, 1990). Few studies have examined the effect of MDMA or other amphetamine analougues (e.g. PCA) in the rat spinal cord. Ridet et al. found that PCA treatment caused a marked and selective elimination of fine 5-HT-IR fibres in the dorsal horn and spared all other larger axons (Ridet et al., 1994). Several studies reported that 5-HT plays an important role in pain perception (Basbaum and Fields, 1984; Hamon, 1997; LeBars, 1988) and the rostral ventromedial medulla, which includes the RMg, is a key neural structure for pain modulation (Marinelli et al., 2002; Mason, 2001; Taylor and Basbaum, 1995) with ON and OFF cells modulating pain inhibition and facilitation (Yang et al., 2006). Besides serotonin there is also a number of neuropeptides coexisting in the RMg, including endogenous opiate peptides, substance P, somatostatin, galanin, vasoactive intestinal polypeptide, neuropeptide Y and calcitonin gene-related peptide (Fields and Basbaum, 1994; Huang et al., 2000).

It is known that the neurotoxins selective for 5-HT neurons (e.g. 5,6-DHT and 5,7-DHT) prevent morphine-induced antinociception (Romandini et al., 1986). In

contrast, one study observed the enhancement of morphine-induced analgesia after repeated injections of MDMA in rats. Furthermore, two weeks after drug treatment the 5-HT content decreased mostly in the cervical portion of the spinal cord and the reduction was smaller in the thoracic segment (Nencini et al., 1988). Our group (Kovacs et al., 2007) found decreased TPH fibre densities in all spinal cord regions 1 week after MDMA administration, although the dorsal horn was the most vulnerable where the fibre density was still lower, albeit statistically not significant at 180 days. Ricaurte et al. demonstrated decreased 5-HT and 5-HIAA concentration in the monkey cervical spinal cord two weeks after MDMA treatment (Ricaurte et al., 1988a). These results indicated that MDMA is toxic to serotonergic fibres not only arising from cells in the DR but also in the descending raphe nuclei. Our data indicate that MDMA treatment influences the descending serotonergic cells and perhaps several physiological functions which are connected to the spinal cord e.g. pain, autonomic function and motor activity as well.

It is well known that serotonergic neurons show a high capacity for axonal sprouting and regeneration after a variety of neuron lesioning techniques, including some using 5,6- and 5,7-DHT (Björklund et al., 1981; Frankfurt and Azmitia, 1984; Wiklund and Mollgard, 1979) in the brain. The serotonergic damage occurring after very high single doses or after successive moderate doses of MDMA has been confirmed across a variety of animal species, including rats, monkeys and other primates. Neuronal recovery is most apparent in rats, whereas monkeys show only partial recovery even after a prolonged period (Ricaurte et al., 2000a). Recovery of serotonergic neurons in most areas examined was observed in animals treated with a single dose of MDMA 180 days previously. Among the exceptions hippocampus should be noted because we found a significant reduction (31-36%) in almost all hippocampal areas 6 months after MDMA treatment. Our results support previous observations that 1 year following MDMA treatment the hippocampus is the only brain region where none of the presynaptic 5-HT neuronal markers recovered (Scanzello et al., 1993). This observation supports previous results that the rate and degree of recovery is region dependent (Lew et al., 1996; Sabol et al., 1996). Our results support previous data showing that the hippocampus is more resistant to recovery compared to other brain regions. The explanation for this might be the specific hierarchy of hippocampal

neurons and/or alteration of synthesis or metabolism of neurotrophic factors (Martinez-Turrillas et al., 2006).

In a previous study (Lew et al., 1996) it was found that 2 weeks after MDMA administration the density of serotonin uptake sites in the cingulate cortex was 20% of that in saline-treated animals, but had significantly increased to 43% of the density of controls by 8 weeks and was completely recovered by 16 weeks. Our data partially support these results because 5-HTT-IR fibre density in the limbic cortical areas was decreased at 21 days but not normalized at 6 months after MDMA.

Among the consequences of these findings might be long-term changes in memory functions and/or anxiety. MDMA-treated rats also displayed impaired memory 3 months after drug administration in an object recognition test (Morley et al., 2001). Indeed, human studies most consistently point to cognitive and memory disturbances in current and previous ecstasy users (Morgan, 2000; Parrott, 2001).

At 180 days we observed a significant reduction in 5-HTT-IR fibre density in the dorsomedial striatum, caudate-putamen, and a trend towards decrease was found in the globus pallidus ($p= 0.068$) and in the ventrolateral striatum ($p=0.053$). In one study (Callaway and Geyer, 1992) investigating 5-HT and 5-HIAA levels in the striatum 36 days after MDMA treatment a significant reduction was observed. Similar results were described earlier (Gurtman et al., 2002; McGregor et al., 2003) 10 and 12 weeks after MDMA administration. Our study supports these results because we found significant decrease in all examined striatal brain areas 21 days after MDMA treatment. Balogh et al. demonstrated a significant decrease (16–20%) in the local cerebral metabolic glucose utilisation in the medial striatum and globus pallidus three weeks after a single MDMA administration in Dark Agouti rats, although change in [^{3}H]paroxetine binding was not significant in this study (Balogh et al., 2004).

High densities of 5-HT_{1B} receptor were found in motor control centers such as the basal ganglia (Sari, 2004). Several studies have suggested that 5-HT_{1B} receptors are involved in several physiological functions, behaviours and psychiatric diseases including control of locomotor activity, drug abuse reinforcement, migraine, anxiety states (Castanon et al., 1997; Green et al., 1984; Griebel et al., 1990) and aggressive behaviour (Flannelly et al., 1985; Kirilly et al., 2006). Previous studies suggested that some of the psychotropic and behavioural effects of MDMA may be mediated by

altered 5-HT_{1B} receptor function. Sexton et al. examined the regulation of rat 5-HT_{1B} receptors after acute and chronic MDMA treatment and observed a transient alteration in striatal 5-HT_{1B} binding sites (Sexton et al., 1999). This study found no change in 5-HT_{1B} mRNA immediately or after 2 weeks, suggesting a posttranslational mechanism of regulation. 5-HT_{1B} receptors appear to mediate MDMA-induced motor hyperactivity: hyperactivity is reduced by antagonists that block 5-HT_{1B} receptors and it is attenuated in 5-HT_{1B} knockout mice (Clark and Neumaier, 2001). Gyongyosi et al. demonstrated a reduced or eliminated AW and diurnal rhythm of this parameter after the administration of the 5-HT_{1B} agonist CP-94,253, in animals treated with MDMA 6 months earlier (Gyongyosi et al., 2008). Parallel MDMA treatment decreased 5-HTT-IR fibre density in several brain areas that are involved in sleep regulation and motor control. In our study the loss of 5-HT nerve terminals in the striatum 6 months after MDMA administration support earlier data that MDMA may cause long-term changes in 5-HT_{1B} receptor function.

Some authors described an increase in serotonergic markers in certain areas (globus pallidus, hypothalamus) several months after the decrease of the axon density which they explained as a „pruning effect". One study provided evidence that 3 of the examined 16 rats showed an altered pattern of distribution of 5-HT axon markers in the hypothalamus in animals treated with MDMA 52 weeks previously (Fischer et al., 1995). In our experiments, we used 6 animals per group, and we did not find any sign of hyperinnervation in any brain areas examined. One possible explanation for the lack of this phenomenon is that the relatively small single dose (15 mg\kg) applied in our study caused only moderate destruction of axons and terminals. Even in the most affected areas the changes did not exceed 30-40%. In previous studies where prunning effect has been shown, higher doses of MDMA were used. Either rats or rhesus monkeys were treated twice a day for 4 consecutive days, thus e.g. for rats a cumulative dose of 160 mg\kg\animal was used (Fischer et al., 1995; Hatzidimitriou et al., 1999).

6.2. Effects of MDMA on sleep

Acute and subacute effects of MDMA on sleep have been described by our group earlier (Balogh et al., 2004). Here we concentrated on chronic and persistent morphological and functional effects of the drug. A selective decrease in REM latency was found 7 and 21 days after MDMA administration. Similarly, an increase in the amount of REM was found in the first hour. None of the other parameters characterizing REM sleep were altered. Our data support the view that initiation of REM sleep is modulated by the serotonergic system (Adrien, 2002; Bhatti et al., 1998; Portas et al., 1996). In contrast, none of the other REM sleep parameters nor most of the NREM sleep parameters were affected by the temporary lesion of the serotonergic system in our study. Sleep disturbances e.g., higher amounts of REM sleep, altered transition from NREMs to REMs have been described in 5-HTT knockout mice (Alexandre et al., 2006; Wisor et al., 2003). It is, however, not known whether a transient decrease of 5-HTT expression together with decreased 5-HT availability in adulthood might cause any significant effect in sleep parameters. It has been suggested that higher amounts of REM sleep, described in 5-HTT knockout mice (Alexandre et al., 2006; Wisor et al., 2003) result from excessive serotonergic tone during early life (Alexandre et al., 2006). Our data support this notion, because a transient decrease of 5-HTT expression together with decreased 5-HT availability observed 21 days after MDMA treatment failed to cause a general enhancement in the amount of REM sleep. Increase in REM sleep was restricted to the first hour of passive phase suggesting that it was caused solely by a decrease in REM latency.

MDMA administration is followed by depletions in 5-HTT-IR fibre density in some, but not all, brain areas involved in the regulation of REM sleep examined in this study. Significant damage was found in the SCN and tuberomamillary nucleus, parallel with reduced REM latency 7 and 21 days after MDMA treatment. In contrast, we could not find significant changes in 5-HTT density around the “effector” neurons located in the medial pontine reticular formation (Sinton and McCarley, 2004) and the peripeduncular tegmental nucleus (Adrien, 2002) in the brainstem, thus, it is unlikely that the observed decrease in REM latency could be a consequence of changes in the serotonergic system of these areas.

The 5-HTT-IR fibre densities were also diminished in the tuberomamillary nucleus which is also an important part of the arousal system. The histaminergic TMN, parallel with the dorsal raphe serotonergic neurons, fire fastest during wakefullness, slow down with EEG during NREM sleep, and nearly stop firing during REM sleep (Saper et al., 2001; Steininger et al., 1999; Strecker et al., 2002). There is evidence that the serotonergic system activates the histaminergic neurons (Eriksson et al., 2001; Saper et al., 2001) thus the decrease of 5-HTT-IR fibre densities in the TMN may indicate a reduced 5-HT excitation on histaminergic TMN neurons which may result in decreased NREM and possibly REM latencies.

The decrease in REM latency induced by MDMA may be explained by a decreased inhibition of the SCN by 5-HT, due to an earlier activation of SCN. Serotonin inhibits the spontaneous discharge of SCN neurons *in vivo* and in hypothalamic slices *in vitro* (Yu et al., 2001). This effect may be mediated via the 5-HT_7 and 5-HT_{1B} receptors (Yannielli and Harrington, 2004; Yu et al., 2001). The SCN strongly promotes REM sleep tendency during the rest phase, and the SCN neurons show increased activity during REM sleep compared to NREM sleep, the peak of the firing rate of SCN is at the middle of the passive phase when the amount of REM sleep is the highest during the day (Deboer et al., 2003; Wurts and Edgar, 2000). In conclusion, the decrease in 5-HTT-IR fibre density in the SCN could be one possible explanation for the decreased REM latency.

MDMA has been shown to disrupt the circadian cycle in vivo and the firing patterns of SCN in hypothalamic slices (Balogh et al., 2004; Colbron et al., 2002; Dafters and Biello, 2003). Furthermore, disruption of the regular cycle has been connected to serotonergic mechanisms (Biello and Dafters, 2001; Colbron et al., 2002). Our findings of altered 5-HTT densities in the SCN after MDMA treatment further support the role of SCN in the sleep disturbances caused by MDMA.

The importance of our findings is further supported by the fact that disturbances of sleep are typical for most depressed patients and belong to the core symptoms of depression according to DSM-IV (Adrien et al., 1991; Akiskal et al., 1982; Riemann et al., 2001). More than 90% of depressed patients complain about disturbances in sleep quality (Mendelson et al., 1977; Riemann et al., 2001). Difficulties in falling asleep, frequent nocturnal awakenings and early morning awakening are the most frequent

complaints, and reduced REM latency and increased sleep fragmentation are the most characteristic alterations described in depression using EEG sleep polygraphy (Riemann et al., 2001). Kupfer et al. also reported the changes in sleep quality in NREM and problems with sleep continuity (Kupfer et al., 1984). Based on these data the hypofunction of the serotonergic system in depression has been suggested (Adrien, 1999). However, it is not known whether these alterations are also connected to decreased availability of 5-HT in the brain.

Despite the reduced REM latency, we could not find significant changes in the amount of REM sleep after REM onset. The firing rate of the DR neurons is almost absent and is also decreasing in the MR during REM sleep (McGinty and Harper, 1976; Portas et al., 1996; Urbain et al., 2006). It is more likely that during REM latency, before the onset of REM sleep, the MDMA-induced damage elicits a faster reduction of the 5-HT input, thus, the threshold will be reached earlier. The amount of REM sleep does not change after that since the 5-HT input is absent. Furthermore, the amount of REM sleep, once initiated, is determined primarily by homeostatic mechanisms (Wurts and Edgar, 2000).

Among the nine parameters of NREM sleep, only three were altered by MDMA treatment and even these alterations failed to follow the time-course of serotonergic damage. The most consistent effect was an increase in delta power during the first hours of the passive phase in NREM sleep. In addition, SWS-1 in the passive phase was decreased at 21 days. This finding is consistent with the increase in delta power caused by 5-HT_{2A} receptor antagonists (Dugovic et al., 1989; Monti and Monti, 1999; Sebban et al., 2002; Ursin, 2002). Interestingly, 5-HT_{2B} and 5-HT_{2C} receptor antagonists fail to cause a similar effect (Kantor et al., 2002; Popa et al., 2005). The present experiments provide evidence that a partial lesion of the serotonergic system depletes the endogenous 5-HT tone that physiologically inhibits delta activity during NREM sleep. The role of tonic inhibition of delta power by 5-HT through 5-HT_{2A} receptors has been suggested on the basis of pharmacological studies (Kantor et al., 2002; Kitka and Bagdy, 2008; Monti and Monti, 1999; Sebban et al., 2002; Ursin, 2002).

A decrease in spontaneously expressed NREM sleep was found in knock out mice lacking 5-HT_{2A} receptors (Popa et al., 2005). The decrease in SWS-1 found in our study is consistent with these data. Since absolutely no decrease in SWS-1 was found at

day 7 despite of an extensive decrease in serotonergic axon density, this latter effect could be considered an adaptive change due to the lack of appropriate tonic activation of 5-HT_{2A} receptors.

NREM disturbances, like increased sleep latency, diminished deep slow wave sleep and changes in wake parameters described in depression (Kupfer et al., 1990; Naylor et al., 1990; Thase et al., 1995) were not found in MDMA-treated animals. Furthermore, the increase in delta power in MDMA-treated rats was just opposite to attenuation of the same parameter reported in some depressed patients (Borbely et al., 1984; Kupfer et al., 1984). Thus, NREM disturbances observed after partial lesion of serotonergic axons are markedly different compared to those described in depressed patients.

Sleep fragmentation, an inverse measure of sleep continuity, was increased by 7 days after MDMA administration. Interestingly, this disturbance was normalized by day 21, despite of the continuous presence of serotonergic lesions in all brain areas.

In one study, sleep alterations were compared in MDMA users and control persons. Significant differences were found only in NREM sleep parameters. REM latency was lower by 20% in MDMA users, but the difference was not significant. The lack of significantly decreased REM latency in MDMA users may be explained by the fact that the time elapsed since the last ecstasy use was very heterogeneous in the sample (Allen et al., 1993).

6.3. Acute and long-term effects of MDMA on aggression

Acute MDMA treatment decreased aggressive-type behaviour as measured by the resident-intruder test. Significant long term decreases in 5-HTT-IR fibre density was found in many brain areas including those involved in the regulation of aggression 21 days after MDMA administration, although at this time-point aggressive-type behaviour was unaltered.

The data from the present study support previous observations that acute MDMA treatment decreases aggressive-type behaviour in rats, as measured by the resident-intruder test (Miczek and Haney, 1994; Navarro and Maldonado, 1999; Verheyden et al., 2002), as well as in human abusers (Curran et al., 2004). However, in

the present study there were some differences in the effects of MDMA upon separate parameters of aggression, for example in the frequency of biting, boxing and kicking. Closer examination of the data reveals that this is mainly due to differences in the manifestation of attack between individuals, with some animals preferring to kick, while others showing a greater tendency to box or bite.

Interestingly, changes were also found in other parameters not closely related to aggression. Introducing an intruder to the home cage usually results in marked increases in locomotor activity and exploratory behaviour from the resident animal, but this hyperlocomotion was significantly attenuated by acute MDMA treatment (**Figure 32**). Similarly, significant decreases were found in social behaviour (**Figure 31**). Thus it appears that if the resident animal is treated with MDMA in advance of the introduction of the intruder, it is less affected by the presence of the intruder animal. In parallel studies (Kirilly et al., 2006) in animals without the behavioural paradigm, that is where no stimulus or target for aggressive behaviour is available, MDMA-induced increases in local cerebral metabolic rate were found in several brain regions that regulate (either activate or inhibit) aggression, but also in other regions that regulate many other physiological functions. This is perhaps not surprising given the widespread anatomical distribution of serotonergic projections, and the possible involvement of both dopaminergic and noradrenergic systems in the pharmacological response to MDMA.

Significant decreases in 5-HTT-IR fibre density between 30 and 40% were found in several brain areas 21 days after a single exposure to MDMA. This finding is totally in agreement with previous data, which showed that a similar MDMA treatment caused long term serotonergic depletion in the forebrain of Dark Agouti rats (Colado et al., 1998; O'Shea et al., 1998). Of particular interest in the context of our behavioural data is the observation of decreased density in some areas of the brain known to be involved in the regulation of aggression and impulsivity. However, despite the deleterious effect of MDMA on the serotonergic system, and previous evidence that depletion of serotonin in the amygdala facilitates aggressive behaviour (Morgan, 2000), the present study indicates that manifestations of aggressive behaviour was not abnormal in rats pre-treated with MDMA 21 days earlier. For example the animals did not exhibit attacks to vulnerable areas on the target intruder, which is considered a pathological form of aggression (Halasz et al., 2002a; Halasz et al., 2002b). There was,

however, evidence from metabolic mapping studies (Ando et al., 2006; Kirilly et al., 2006) that, in the resting state at least, MDMA pre-treatment did result in altered functional activity in some elements of the amygdala, indicating that the treatment was not without effect.

The lack of evidence for increased aggression in this test may be explained in many ways. First, the level of aggression exhibited by rats in this test is normally relatively high. It could be difficult to detect further small increases in an already high aggressive state, which is described as an essential factor in this test paradigm (Navarro and Maldonado, 1999). Furthermore, chronic social isolation, which is necessary to confer resident status, has been reported to decrease serotonin levels in forebrain areas in the long-term (Dalley et al., 2002; Fulford and Marsden, 1998; Robbins et al., 1996), and indeed this could be one explanation for the increased baseline aggression in the resident-intruder test. A further decrease in 5-HT content as a result of MDMA pre-treatment may not elicit any further increase in aggression.

Moreover, in keeping with these behavioural findings, LCMRglu was significantly decreased (Kirilly et al., 2006) 3 weeks after MDMA administration, albeit in the absence of aggression-inducing stimuli. A decrease in metabolism was found in several brain areas, including also those thought to mediate aggressive behaviour, but also in those that inhibit aggression. It is conceivable that the integration of these inhibitory and disinhibitory effects could confound our measures of aggression, or alternatively represent a compensatory mechanism to normalise function.

Some studies have demonstrated an increase in aggression and impulsivity in heavy ecstasy users (Bond et al., 2004; Gerra et al., 2000; Gerra et al., 1998) which may suggest that in the long term MDMA might increase aggression in humans. However, no significant increase was found in light/moderate users (Morgan, 1998; Parrott et al., 2000) and earlier studies in humans have found that, although aggression increases 4 days after exposure to MDMA, there is no significant increase in aggressive behaviour 7 days after the exposure (Curran et al., 2004). Only McCann et al. reported that heavy ecstasy users had lower aggression than control participants (McCann et al., 1994).

Our results indicate that all of the acute behavioural effects of MDMA were preserved when a neurotoxic MDMA pretreatment was applied 3 weeks before the test. The MDMA-induced depletion of serotonergic terminals, for which our

immunohistochemistry data provide positive evidence in our study, could be assumed to reduce the availability of releasable 5-HT, and might be expected to result in an attenuated anti-aggressive response to acute MDMA treatment. However, these anti-aggressive effects or any possible changes caused by serotonin depletion, may possibly be masked by the general inhibitory behaviour which is unaffected by neurotoxic MDMA pretreatment.

In our other parallel study (Kirilly et al., 2006) we investigated the effects of two 5-HT_{1B} agonists (CGS-12066A and CP-94,253, both 5 mg/kg i.p.) on aggressive behaviour in drug-naive rats and also in rats exposed to MDMA. We found that the anti-aggressive effects of 5-HT_{1B}-agonists in MDMA-pretreated rats were, in general, preserved and only modest changes were observed. CGS-12066A, CP-94,253 produced marked decreases in aggressive behaviours, especially in biting, boxing and kicking found in drug-naive rats. These studies provide evidence that aggressive behaviour and acute anti-aggressive effects of 5-HT_{1B} receptor agonists remain intact 3 weeks after a single dose of the drug despite significant damage to the serotonergic system. These data do not, therefore, indicate a significant sensitization of 5-HT_{1B} receptor function as a consequence of a long-term serotonergic depletion. 5-HT_{1B} receptors involved in this action may be located post-synaptically (Barnes and Sharp, 1999; Clark and Neumaier, 2001; Scearce-Levie et al., 1999) or are heteroreceptors, located not only on serotonergic terminals, but also on terminals where they might inhibit the release of other neurotransmitters including GABA, acetylcholine or glutamate (Clark and Neumaier, 2001; Sexton et al., 1999). Furthermore, there is the possibility that non-serotonergic mechanisms are also involved in the acute effects of MDMA upon aggression, including dopamine, glutamate or GABA (Sexton et al., 1999). It is also possible that anxiety, reactivity and impulsivity confound the overt behavioural expression of MDMA-induced effects, as has previously been described after partial 5-HT depletion (Green et al., 2003; Harro, 2002). There was, however, a difference in drug-induced motor activation. The increase in locomotor activity and exploration was significantly enhanced in MDMA pretreated rats, suggesting that the involvement of 5-HT_{1B} receptors in locomotor activation differs from that in the regulation of aggression and impulsivity.

7. CONCLUSIONS

The results of our study, which has focused on the long-term neurotoxic effects and recovery after a single dose of MDMA, lead us to the following conclusions:

1. Our findings concerning the changes detected in 5-HTT mRNA expression and fibre density both indicate lasting impairment of the serotonergic system and suggest that a single use of MDMA may be associated with long-lasting cognitive, learning, memory and mood deficits and sleep disturbances.
2. Previous studies found that several amphetamine derivatives are more toxic to "fine" 5-HT fibres derived from the DR than "beaded" 5-HT fibres derived from the MR. However, later neuroanatomical and biochemical studies suggest that DR-selectivity of these amphetamines may not be exclusive. In our study the effects of MDMA on 5-HTT mRNA expression in the DR and MR did not differ in time or extent. Our immunohistochemical data also support the conclusion that axons of median raphe origin are also affected by MDMA. These results do not support the view that only „fine" axons derived from the DR are damaged by the drug.
3. Our results indicate that different brain areas are damaged to differing degrees after MDMA administration. This observation supports previous data that the rate and degree of recovery is region-dependent and the hippocampus is more resistant to recovery compared to other brain regions. The explanation for this might be the specific hierarchy of hippocampal neurons and/or alteration of synthesis or metabolism of neurotrophic factors. Among the consequences of these findings may be long-term alterations and disturbances in cognitive and memory functions in previous ecstasy users.
4. Our results support the view that initiation of REM sleep and delta power NREM sleep are modulated by the serotonergic system. MDMA administration is followed by depletions in 5-HTT-IR fibre density in some, but not all, brain areas involved in the regulation of REM sleep examined in this study.

5. Altered sleep functions were found after a single dose of MDMA. Some of these (e.g. reductions in REM latency and sleep fragmentation) were consistent with those described in depression. Effects on other (e.g., NREM) parameters failed to resemble disturbances described in depression. We may conclude that all sleep disturbances described in depression can not be explained by this type of serotonergic damage or the changes in the serotonergic system in depression are only partially similar to the effects of MDMA, and thus point to the involvement of other alterations or the involvement of additional neurotransmitter systems in the pathophysiology of depression.
6. Acute MDMA administration abolished aggressive behaviour in the resident-intruder test as expected. Unexpectedly, MDMA pretreatment and the consequent partial depletion of serotonergic terminals did not increase aggression.
7. Our results draw attention to the long-term dangers of ecstasy use particularly in poor metabolizer persons.

NEW ORIGINAL OBSERVATIONS

1. To our knowledge, our group was the first to examine 5-HTT mRNA expression in the brainstem and medullary raphe nuclei after a single dose of MDMA. We were the first to show that the effects of MDMA on 5-HTT mRNA expression in the dorsal and median raphe nucleus do not differ in time or extent.
2. Our group was the first to publish that after MDMA administration the 5-HTT mRNA expression shows a transient increase followed by a transient decrease and a complete recovery during the 180-day period in the examined raphe nuclei.
3. Our group was the first to carry out such a detailed and long term immunohystochemical study concerning the effects of MDMA. Our studies included almost all brain areas. Based on the results from the examined 32 brain areas we conclude that 7 and 21 days after MDMA treatment the 5-HTT-IR fibre density decreases and at 180 days there is regeneration, except for a few brain areas.
4. In the literature we were first to characterize the chronic sleep effects of MDMA. We measured 16 sleep parameters in 3 different time-points. The most important results are reduced REM latency, increased sleep fragmentation and the changes in the EEG delta power density.
5. We were first to examine the effects of MDMA on sleep and aggression parallel with the changes in 5-HTT-IR fibre density in brain areas relevant to sleep and aggression.

8. SUMMARY

8.1. Summary

"Ecstasy", 3,4-methylenedioxymethamphetamine (MDMA), an amphetamine analogue is one of the most widely used recreational drugs. In spite of the fact that neurotoxic effects of MDMA has been found in several species from rodents to non-human primates, and results increasingly point to damage also in human MDMA users, data about the sensitivity of different brain areas and the recovery after neuronal damage are scarce. Serotonin transporter (5-HTT) mRNA in the raphe nuclei also has not been examined; consequently, direct comparisons of responses of dorsal and other raphe nuclei have not been measured.

We specifically emphasized the effects of MDMA on 5-HTT protein in our research because previous studies have shown that MDMA selectively destroyed the serotonergic system both in experimental animals and humans. This protein is target of MDMA and also a marker of the established damage.

Humans with genetic predisposition for the slow metabolism of MDMA, the so-called "poor metabolizers" of debrisoquin are at higher risk. Five- 9% of the Caucasian population is considered to carry this phenotype. These studies were carried out in Dark Agouti rats, a special strain that show decreased microsomal CYP2D1 isoenzyme activity, and thus may serve as a model of vulnerable human users.

These works were designed to characterize MDMA-induced damage and recovery of the serotonergic system including sleep and morphological changes within 180 days. In our experiments we investigated the 5-HTT mRNA expression in the brainstem (dorsal and median raphe nucleus) and medullary (nucleus raphe magnus, obscurus and pallidus) raphe nuclei, 5-HTT immunoreactive (IR) fibre densities in several brain areas, and 16 functional measures of sleep in response to a single dose of +/- MDMA (15 mg\kg) which is relevant to ecstasy use in humans. Studies were carried out at least at three time-points within 180 days. Furthermore, behavioural experiments were performed 21 days after MDMA treatment, because at this timepoint morphological studies indicated significant axonal damage without any sign of regeneration.

We found similar changes in 5-HTT mRNA expression in the examined raphe nuclei, namely transient increases 7 days after MDMA treatment followed by transient decreases at 21 days. Significant (20–40%), widespread reductions in 5-HTT-IR fibre density were detected in most brain areas at 7 and 21 days after MDMA administration. All cortical, but only some brainstem areas were damaged.

Parallel to the neuronal damage we observed significant reductions in rapid eye movement (REM) sleep latency, increased fragmentation of sleep and increases in delta power spectra in non-REM sleep. Some of these (reductions in REM latency and increased sleep fragmentation) were consistent with those described in depression. At 180 days almost all functional changes in sleep were normalized together with 5-HTT mRNA expression in the examined raphe nuclei and the recovery of 5-HTT-IR fibre density in most brain areas. Among the exceptions hippocampus should be noted because we found a significant reduction even 180 days after MDMA treatment. Disturbances in cognitive and memory functions in previous ecstasy users could be likely consequences of the long-term damage of the hippocampus. Our results also suggest that the acute MDMA administration abolished aggressive behaviour but MDMA pretreatment and the consequent depletion of serotonergic terminals did not affect aggression.

Our findings concerning the changes detected in 5-HTT mRNA expression and fibre density indicate lasting impairment of the serotonergic system and suggest that a single use of MDMA may be associated with long-lasting cognitive, learning, memory and mood deficits and sleep disturbances particularly when a constellation of genetic vulnerability and certain environmental factors are present. Our data provide further evidence for the connection between altered serotonergic functions and sleep disturbance.

8.2. Összefoglalás

A rekreációs drogok körébe tartozó 3,4-metiléndioxi-metamfetamin (MDMA), közismert nevén az „ecstasy” használata a hazai és európai fiatalok körében egyre nő. Kutatásom témája az MDMA neuronkárosító hatásának részletes leírása. Annak ellenére, hogy az MDMA neuronkárosító hatását a rágcsálóktól a majmokig számos fajban kimutatták, és egyre több adat szól amellett, hogy ennek veszélye a drogot fogyasztó személyek között is fennáll, kevés adatot találunk a különböző agyterületek eltérő érzékenységéről, szinte alig rendelkezünk információval a neuronkárosodást követő regenerációról, és semmit sem tudunk a raphe magokban található szerotonerg sejtek válaszáról.

Kísérleteimben különös hangsúlyt kapott a szerotonin transzporter (5-HTT) fehérjére gyakorolt hatások vizsgálata, mert az irodalom alapján az MDMA mind állatkísérletekben mind humán vizsgálatokban jelentősen és szelektíven károsítja a szerotonerg rendszert és ez a fehérje egyrészt célpontja is az MDMA-nak, másfelől markere is a létrejött károsodásnak.

Az MDMA lebontásáért a debrizoquin-4-hidroxiláz vagy CYP2D6 enzim felelős. Az európai népesség 5-9%-ában ennek az enzimnek genetikailag meghatározottan csökkent a működése, melynek következtében ebben a populációban feltehetőleg nagyobb az MDMA toxicitása. Kutatásaink során Dark Agouti patkánytörzset alkalmaztuk, mely mintegy modellje a genetikai okok miatt lassan metabolizáló humán fenotípusnak.

Vizsgálatainkban a humán hatások szempontjából releváns egyszeri dózis +/- MDMA (15 mg/kg) kezelést követően párhuzamosan vizsgáltuk a 5-HTT mRNS expresszióját az agytörzsi (nucleus raphe dorsalis és medianus) és nyúltvelői (nucleus raphe magnus, obscurus és pallidus) raphe magokban, a 5-HTT tartalmú rostok sűrűségét számos agyterületen és 16 alvásparamétert a kezelést követő fél éves időszak alatt, míg a szerotonerg rendszer károsodik, majd részben regenerálódik. Mivel a vizsgált morfológiai paraméterekben 21 napnál igen markáns változást találtunk, ugyanakkor a regenerációnak még semmi jelét sem láttuk, ebben az időpontban magatartás vizsgálatot is végeztünk.

Kísérleteink alapján az agytörzsi, valamint a nyúltvelői raphe magokban hasonló változásokat találtunk a 5-HTT mRNS expressziójában, nevezetesen a 7 nappal a beadást követően mért átmeneti növekedést egy átmeneti csökkenés követi 21 nappal az MDMA kezelés után. A 5-HTT fehérje mennyisége a legtöbb általunk vizsgált agyterületen jelentős (20-40%) csökkenést mutatott. A kérgi területek mind, az agytörzsi területek közül azonban csak némelyik sérült.

Ezzel párhuzamosan a vizsgált alvásparaméterek közül szignifikánsan csökkent a REM (paradox alvás) latencia, fokozódott az alvástöredezettség, míg a non-REM alvás idején az ún. delta EEG teljesítmény-sűrűség fokozódott. Ezek közül az első két hatás egybeesik azzal, amit depressziós betegeken leírtak. Hat hónappal a kezelés után az 5-HTT mRNS expressziója az összes vizsgált raphe magban, valamint az 5-HTT rostdenzitás szinte az összes általunk mért agyterületen normalizálódott, párhuzamosan az alvásparaméterekkel. A néhány kivétel közül kiemelendő a hippocampus, ahol még fél év után is megmaradt a csökkenés. A hippocampus tartós károsodása valószínűleg összefügg azzal az adattal, hogy a krónikus ecstasy fogyasztókban kognitív- és memóriaproblémák jelentkezhetnek. A magatartás vizsgálatban kapott eredményeink szerint az MDMA akut hatása, hogy csökkenti az agressziót. Ugyanakkor a később kialakult neuronkárosodás nem okozott jelentős változást az agresszió mértékében.

Mivel az 5-HTT mRNS expressziójában és a rostdenzitásban talált változások egyaránt a szerotonerg rendszer tartós károsodására utalnak, a genetikailag vagy az egyéb körülmények miatt lassan metabolizáló személyek már egyszeri fogyasztás után is komoly veszélynek vannak kitéve, amely számos funkció - pl. a tanulás, memória, hangulat, alvás-ébrenlét ciklus - zavarához, egyensúlyának felbomlásához vezethet. Eredményeink alátámasztják az összefüggést a szerotonerg rendszer változása és az alvásproblémák között.

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10. PUBLICATIONS

10.1. **Publications relevant to the dissertation**

10.1.1. Journal articles

1. Kirilly E., Molnar E., Balogh B., Kantor S., Hansson S. R., Palkovits M., Bagdy G.: **Decrease in REM latency and changes in sleep quality parallel serotonergic damage after MDMA: a longitudinal study over 180 days.** The International Journal of Neuropsychopharmacology (2008), 11: 795-809. IF: 4.895

2. **Kirilly E.,** Benko A., Ferrington L., Ando R.D., Kelly P.A.T., Bagdy, G., **Acute and long-term effects of a single dose of MDMA on aggression in Dark Agouti rats.** The International Journal of Neuropsychopharmacology, (2006), 9(1):63-76. IF: 5.184

3. Ferrington L., **Kirilly E.**, McBean D.E., Olverman H.J., Bagdy G., Kelly P.A.T.: **Persistent cerebrovascular effects of MDMA and acute responses to the drug**, European Journal of Neuroscience, (2006), 24: 509-519. IF: 3.709

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10.1.2. Posters and presentations

7. **Kirilly E.**, Ando R.D., Palkovits M., Bagdy G.: **MDMA (Ecstasy) causes long-term changes in the expression of the serotonin transporter.** 11th of Congress of the Hungarian Society of Neuroscience, Pécs, Hungary, January 26-29, 2005, Clinical Neuroscience 58, 1. különszám, P. 50, 2005

8. **Kirilly E.**, Ando R.D., Palkovits M., Bagdy G.: **Serotonin transporter mRNA expression in raphe nuclei after MDMA treatment.** Spring Symposium of the Hungarian Society for Experimental and Clinical Pharmacology, June 6-7 2005, Budapest, Hungary, Inflammopharmacology Vol. 13, No. 5-6, pp 501.

9. **Kirilly E.**, Palkovits M., Bagdy G.: **Effects of ecstasy on serotonin transporter in the rat brain**, 8th Congress of Hungarian Association of Psychopharmacology, October 6-8 2005, Tihany, Hungary, Neuropsychopharmacologia Hungarica, VII, Suppl.1, 2005, pp. 37-38.

10. **Kirilly E.**, Palkovits M., Bagdy G.: **Time-course of molecular events of MDMA-induced neuronal damage in the serotonergic system**, Semi-centennial conference of Semmelweis University, October 12-14, 2005, Budapest, Hungary, Pharmacy: Smart Molecules for Therapy: Programme book of abstracts, P-52

11. **Kirilly E.**, Tóth Z.E., Gerber G., Palkovits M., Bagdy G.: **3,4-methylenedioxymethamphetamine (MDMA) modulates serotonin transporter mRNA expression in the rat brainstem**, Society for Neuroscience 35th Annual Meeting, November 12-16 2005, Washington DC, P111.12

12. **Kirilly E.**, Palkovits M., Bagdy G.: **Egyszeri MDMA („ecstasy") kezelést követő tartós szerotonerg idegkárosodás Dark Agouti patkányban**, PhD Tudományos Napok 2006, Budapest, 2006.április 13-14.

13. **Kirilly E.**, Steinbusch H.W.M., Palkovits M., Bagdy G.: **Damage and recovery after a single dose of ecstasy: a 6-months follow-up study**, CINP 2006 Chicago Congress, Chicago, 9-13 July 2006, The International Journal of Neuropsychopharmacology, Vol. 9, Suppl. 1, July 2006, P02.019

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16. **Kirilly E.**, Molnar E., Balogh B., Katai Z., Palkovits M., Bagdy G.: Neuronal damage and recovery induced by a single dose of MDMA: morphological studies correlation with sleep parameters, ECNP, AEP Seminar in Neuropsychopharmacology, April 10-12 2008, Siofok, Hungary

17. **Kirilly E.**, Molnar E., Katai Z., Pap D., Harry W.M. Steinbusch, Palkovits M., Bagdy G.: Diurnal rhythm and sleep parameters during neuronal damage and recovery after (+/-) 3,4-methylenedioxymethamphetamine (MDMA), XXVI. CINP Congress, Münich, 13-17 July 2008, The International Journal of Neuropsychopharmacology, Vol. 11, Suppl. 1, July 2008, P11.16

10.2. Other publications

10.2.1. Journal articles

18. Tóth Z., Zelena D., Mergl Z., **Kirilly E.**, Várnai P., Mezey E., Makara G., Palkovits M.: **Chronic repeated restraint stress increases prolactin-releasing peptide/tyrosine-hydroxylase ratio with gender-related differences in the rat brain.** Journal of Neurochemistry (2008), 104(3):653-66 IF (2007): 4.451

19. Heinzlmann A., **Kirilly E.**, Meltzer K., Szabó E., Baba A., Hashimoto H., Köves K.: **PACAP is transiently expressed in anterior pituitary gland of rats: in situ hybridization and cell immunoblot assay studies.** Peptides (2008), 29(4):571-7 IF(2007): 2.368

10.2.2. Posters and presentations

20. Ando R.D., Adori C., **Kirilly E.**, Moszkovkin G., Kovacs G.G., Bagdy G.: **Persistent neurotoxic effects of a single dose of MDMA (Ecstasy) on serotonergic axons**. 11th of Congress of the Hungarian Society of Neuroscience, Pécs, Hungary, January 26-29, 2005. Clinical Neuroscience 58, 1. különszám, P. 6, 2005

21. Ando R.D., Adori C., **Kirilly E.**, Moszkovkin G., Kovacs G.G., Bagdy G.: **Persistent impairment of serotonergic axons after a single dose of MDMA (Ecstasy).** Spring Symposium of the Hungarian Society for Clinical and Experimental Pharmacology, June 6-7 2005, Budapest, Hungary, Inflammopharmacology Vol. 13, No. 5-6, pp 501.

22. Bagdy G., Kovacs G.G., Adori C., Ando R.D., **Kirilly E.**.: **Behavioural consequences of ecstasy-induced damage of serotonergic neurons: focus on anxiety and impulsivity**, 8th Congress of Hungarian Association of Psychopharmacology, October 6-8 2005, Tihany, Hungary, Neuropsychopharmacologia Hungarica, VII, Suppl.1, 2005, pp. 13.

23. Ando R.D., Adori C., Kovacs G.G., **Kirilly E.**, Palkovits M., Bagdy G.: **Serotonergic axonal damage in the spinal cord after a single dose of Ecstasy (MDMA, 3,4-methylenedioxymethamphetamine)**, **International IBRO Workshop Budapest, Hungary, 26-28 January 2006**, Clinical Neuroscience, P-165

24. **Kirilly E.**, Gonda X., Andó R.D., Bagdy G.: **Drogok tisztasága a feketepiacon: az ecstasy tabletták összetétele**, CPhH XIII. Budapest, 2006. május 25-27.

25. Ando R.D., Adori C., **Kirilly E.**, Kovacs G.G., Benko A., Bagdy G.: **Fluoxetine-induced acute anxiogenic effects is attenuated six months after a serotonergic damage caused by MDMA despite recovery of neocortical areas but not hippocampus**, 9th Congress of Hungarian Association of Psychopharmacology, October 5-7 2006, Tihany, Hungary, Neuropsychopharmacologia Hungarica, VIII, Suppl.2, 2006, pp. 15.
26. Gonda X., **Kirilly E.**, Bagdy G.: **Gyógyszerválasztás genetikai markerek alapján: A szerotonin transzporter gén és a depresszió összefüggései,** CPhH XIII. Budapest, 2006. május 25-27.

27. Toth Z.E., Zelena D., Mergl Z., **Kirilly E.**, Borhegyine Holderith N., Makara G. B., Palkovits M.: **Changes in prolactin-releasing peptide mRNA expression int he brain of male and female rats after chronic restraint stress**, Society for Neuroscience 35th Annual Meeting, November 12-16 2005, Washington DC, P639.12

28. Toth Z.E., Zelena D., Mergl Z., **Kirilly E.**, Borhegyine Holderith N., Makara G. B., Palkovits M.: **Effect of repeated restraint stress on prolactin-releasing peptide mRNA expression in the rat brain, International IBRO Workshop Budapest, Hungary, 26-28 January 2006**, Clinical Neuroscience, P-95

29. Bagdy György, **Kirilly Eszter**, Katai Zita, Gonda Xenia, Kitka Tamas, Balogh Brigitta: **Az antidepresszívumok hatásaiban mutatkozó különbségek összefüggése a szerotonin transzporter genetikai és neurokémiai eltérésével,** 10th Congress of Hungarian Association of Psychopharmacology, October 4-6 2007, Tihany, Hungary, Neuropsychopharmacologia Hungarica, IX. Suppl.3, 2007, pp. 16-17.

30. Katai Zita, Balogh Brigitta, Kitka Tamás, **Kirilly Eszter**, Bagdy György, **Acute effects of citalopram on REM sleep in control and MDMA pretreated rats using cosinor analysis,** 10th Congress of Hungarian Association of Psychopharmacology, October 4-6 2007, Tihany, Hungary, Neuropsychopharmacologia Hungarica, IX. Suppl.3, 2007, pp. 31-32.

31. Papp R.S., **Kirilly E**., Kitka T., Bagdy G., Szabó-Meltzer K., Tóth Z. E., **Prolactin-Releasing Peptide Expressing Neurons in the Dorsomedial Nucleus of the Hypothalamus Influence the Orexinergic System via Direct Connections with MCH and Orexin Producing Cells,** PhD Tudományos Napok, Budapest, April 10-11 2008

32. Ando R.D., Adori C., **Kirilly E.**, Kovacs G.G., Benko A., Bagdy G.: **Fluoxetine-induced acute anxiogenic effect is attenuated six months after a serotonergic damage caused by MDMA despite recovery of neocortical areas but not hippocampus**, ECNP, AEP Seminar in Neuropsychopharmacology, April 10-12 2008, Siofok, Hungary

33. Katai Z., Garay T., Balogh B., Kitka T., **Kirilly E.**, Bagdy G.: **Altered sleep effects of acutely administered citalopram after partial serotonergic damage by MDMA**, ECNP, AEP Seminar in Neuropsychopharmacology, April 10-12 2008, Siofok, Hungary

34. Heinzlmann A., **Kirilly E.,** Meltzer K., Szabó E., Akemichi B., Hitoshi H., Köves K. A PACAP átmenetileg expresszálódik a hypophysis mellső lebenyében: in situ hybridizációs és cell immunoblot assay (ciba) tanulmány, Magyar Endokrinológiai és Anyagcsere Társaság XXII. Kongresszusa, 2008 június 5-7., Eger, Hungary

35. Tóth Zs. E, Mergl Zs., **Kirilly E.**, Szabó-Meltzer K., Zelena D. **Elevated PrRP mRNA Expression in Brattleboro Rats Does not Affect the Response to Acute Stress,** 12th Meeting of the Hungarian Neuroscience Society, 22-24 January, 2009 Budapest, Hungary, P038

10.3. Book chapters

36. Gonda X, **Kirilly E.** Az ecstasy története. Fogyasztási szokások a népesség körében. In: Bagdy G. (ed), Amit az ecstasyról tudni kell. Akadémiai Kiadó, Budapest: 2006:11-20.

37. **Kirilly E.**, Lazáry J. Mit tartalmaz az ecstasy tabletta? In: Bagdy G. (ed), Amit az ecstasyról tudni kell. Akadémiai Kiadó, Budapest: 2006: 20-36.

38. **Kirilly E.**, Lazáry J. Az ecstasy mint lehetséges gyógyszer? In: Bagdy G. (ed), Amit az ecstasyról tudni kell. Akadémiai Kiadó, Budapest: 2006:171-177.

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