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Research report

Demonstration and localization of neuronal degeneration in the rat forebrain following a single exposure to MDMA

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Abstract

Methylenedioxymethamphetamine (MDMA, Ecstasy) is a powerful releaser of serotonin. Increasing recreational use of this stimulant and hallucinogenic drug has raised concerns about its potential to produce brain damage. The vast majority of previous research studies have focused on the compound's ability to deplete serotonin (5-hydroxytryptamine, 5-HT) from axon terminals. Despite extensive research on this '5-HT terminal neurotoxicity', a much less studied aspect of MDMA toxicity involves its ability to actually kill nerve cells. Only two prior studies mention the existence of MDMA-induced neuronal degeneration, as reflected by a limited number of argyrophylic neurons within the somatosensory cortex, following very high doses of MDMA. The development of Fluoro-Jade B as a simple and reliable marker of neuronal degeneration has allowed us to conduct the first comprehensive localization of MDMA induced neuronal degeneration throughout the entire rat forebrain. In addition to the previously reported neuronal degeneration within parietal cortex, degenerating neurons were also observed in the insular/perirhinal cortex, the ventromedial/ventrolateral thalamus, and the tenia tecta. The extent of neuronal degeneration observed generally correlated with the degree of hyperthermia achieved.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Methylenedioxymethamphetamine; Ecstasy; Fluoro-Jade B; Pathology; Neuronal degeneration

1. Introduction

The neurotoxic potential of MDMA (methylenedioxymethamphetamine) has been the subject of considerable attention and controversy in recent years, primarily as a result of its widespread illicit use as a psychostimulant. The history of the compound dates back to 1912 when it was first synthesized and subsequently patented by Merck in 1914. The first comprehensive study of its toxicology and behavioral pharmacology was conducted by the University of Michigan under a contract from the Army Chemical Center [26] in 1953. However, this work was not declassified and published until 20 years later [9]. The study found MDMA to be less toxic than MDA (methylenedioxyamphetamine) but more toxic than mescaline. This study reported the LD_{50} in rats to be 49 mg/kg i.p. Certain substituted amphetamines such as *p*-chloroamphetamine [20], MDA [15], and D-fenfluramine [6] were among the first compounds shown to cause semi-chronic serotonin depletion. Subsequent examination of MDMA [21,29] indicated that this compound also has the potential to produce long-term depletion of brain serotonin. The most comprehensive of the early studies [4] used both biochemical and histological endpoints to assess MDMA neurotoxicity. In addition to confirming a drop in serotonin levels, the investigators reported abundant argyrophylic terminal-like puncta in the striatum and somatosensory cortex. Also, they reported argyrophylic neurons within the somatosensory cortex. Follow up studies have focused almost exclusively on MDMA-induced damage to serotonergic axon terminals, and the number of publications on this subject are too numerous for a complete listing of references (for reviews see Refs. [12,18]). This is in contrast to the phenomenon of overt neuronal degeneration within the parietal cortex, which

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was reported in only one subsequent article [10]. The present study was undertaken due to the scarcity of information on this subject, and because of the potential functional consequences of overt neuronal degeneration. Thus, the purpose of the study was to validate the existence of neuronal degeneration in the parietal cortex at more moderate doses using a more modern and reliable marker of neuronal degeneration, and also to resolve whether neuronal degeneration might also occur in forebrain regions other than parietal cortex.

2. Materials and methods

2.1. Animals and treatment

A total of 16 adult male Sprague–Dawley rats were used in this study in accordance with the institutional animal care and use policy. Four rats were allotted per dosing group, with each group receiving one of the following single i.p. doses of MDMA: 0 mg/kg (saline control); 10 mg/kg; 20 mg/kg; and 40 mg/kg. No injection volume exceeded 1 ml. The temperature monitoring procedure has been previously described elsewhere [3]. Briefly, body temperature was measured via a rectal transducer once every hour for 10 h following dosing. Thus, the maximum temperature values listed in Table 2 reflect the highest recorded temperature, although the actual peak temperature may be slightly higher if it occurred between hourly measurements. Following a survival interval of 3 days, the animals were anesthetized with ketamine (75 mg/kg) and xylazine (9 mg/kg) and perfused with 10% neutral phosphate-buffered formalin (4% formaldehyde). Brains were post-fixed and cryoprotected for at least 16 h in the same fixative solution plus 20% sucrose.

2.2. Histology

Brains were cut into 25-μm-thick sections on a freezing microtome and subsequently mounted on gel coated slides (prepared with1% pig skin gelatin (Sigma)) and dried on a slide warmer (at 50 °C for at least half an hour). The sections were then stained with Fluoro-Jade B to allow for the detection and localization of degenerating neurons, as previously described [23]. Briefly, slides were immersed in a solution of basic alcohol (1% potassium hydroxide in 80% alcohol) for 5 min, followed by a 2-min incubation in 70% alcohol, followed by a 2-min rinse in distilled water. Sections were then incubated for 10 min in a 0.06% solution of potassium permanganate to reduce background staining. Slides were rinsed briefly in distilled water and stained for 20 min in a 0.0004% solution of Fluoro-Jade B (Histo-Chem, Jefferson, AR) dissolved in a vehicle of 0.1% acetic acid. Slides were then rinsed for 1 min in each of three changes in distilled water, the excess water was drained, and the slides were then air dried on a slide warmer (typically 5–10 min). The sections were then xylene cleared (at least 1 min), coverslipped with D.P.X. non-fluorescent mounting media (Fluka/Sigma) and examined with an epifluorescent microscope using blue light excitation. The same filter cube recommended for visualizing FITC was used. All sections were visually inspected using a 20× objective. Fluoro-Jade B-positive cells were plotted on brain section templates from Paxinos and Watson's atlas of the rat brain [14]. Brain regions exhibiting conspicuous degeneration were also photographed at both high and low magnification. Occasionally the Fluoro-Jade labeling was counterstained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) to produce a fluorescent Nissl stain of normal cells. This was accomplished by incorporating 0.0002% DAPI with the Fluoro-Jade staining solution. This tracer was visualized using ultraviolet light excitation. Double exposures (combined blue/ultraviolet light excitation) were used to simultaneously visualize both tracers.

3. Results

3.1. Graphic summaries of results

The results of this study are summarized in several formats. The number of animals exhibiting lesions in specific brain structures at various doses can be found in Table 1. This table reveals that all animals receiving 40 or 20 mg/kg of MDMA exhibited relatively extensive and widespread neuronal degeneration. Only one of the four animals receiving 10 mg/kg MDMA exhibited any neuronal degeneration, while none of the saline injected animals contained any Fluoro-Jade B-positive neurons. Table 2 portrays the exact total number of degenerating cells found in each animal that exhibited neuronal degeneration. The dosage and maximum body temperature is also indicated, showing that all doses result in demonstrable hyperthermia compared to control animals. Concerning those animals that did not exhibit neuronal degeneration, all four control animals had body temperatures of 38.6 °C. Of those animals given the lowest dose of 10 mg/kg, only the animal that achieved a body temperature of 41.8 °C

Table 1
Number of animals with lesions in specific brain structures

Brain structure	Dose			
	40 mg/kg	20 mg/kg	10 mg/kg	0 mg/kg
Parietal cortex	++++	+++	+	
Insular cortex	+++	+		
Tenia tecta	++++	++++	+	
Ventromedial thalamus	++++	+++	+	
Paraventricular thalamus	++++	+++		
Intralaminar thalamus	+++	+++		
Bed nucleus of the stria terminalis	++			

Table 2
Total number of degenerating neurons

	Rat no.									
	MD 14	MD 22	MD 23	MD 15	MD 6	MD 8	MD 19	MD 17	MD 27	Cont.
Dose (mg/kg, i.p.)	40	40	40	40	20	20	20	20	10	0
Max. temp. (°C)	41.4	41.5	41.6	41.4	41.2	41.9	41.8	40.7	41.8	38.6
Parietal cortex	41	53	72	36	9	9		275	6	
Insular cortex	789	700		216				981		
Tenia tecta	74	23	32	12	15	53	34	29	16	
VM/VL thalamus	62	460	286	288	29	143		12	9	
Paraventricular thalamus	2	6	52		41	53		13		
Intralaminar thalamus	18	69	80		68	15		62		
Bed nucleus of stria terminalis		13	46							

VM/VL, ventromedial/ventrolateral.

showed lesions. The other three animals, which did not exhibit neuronal degeneration, had body temperatures of 40.4 °C, 40.1 °C, and 38.9 °C, respectively. Fig. 1 is a schematic plot of animal MD 17 (20 mg/kg) illustrating the relative number and location of degenerating neurons in each region of the forebrain. Fig. 2 is a composite plate that illustrates, with both high- and low-magnification photomicrographs, the brain regions most frequently lesioned by MDMA. These structures include the tenia tecta (Fig. 2A,B), the parietal cortex (Fig. 2C,D), the insular cortex (Fig. 2E,F), ventrobasal thalamus (Fig. 2G), and intralaminar thalamus (Fig. 2H).

3.2. Regional description of pathology

3.2.1. Parietal cortex

Both pyramidal and non-pyramidal Fluoro-Jade B-positive neurons could be observed in layers III and IV of parietal cortex (Fig. 2C,D). The cells extended from approximately the level of the genu of the corpus callosum caudally to the level of the arcuate nucleus of the hypothalamus. Labeled terminal-like puncta could also be observed adjacent to degenerating neurons.

3.2.2. Tenia tecta

This hippocampal remnant was the brain region most consistently vulnerable to MDMA-induced neuronal degeneration (Table 1). Degenerating neurons were found in the tenia tecta of all affected animals. The labeled granule cells were small with monopolar medially extending dendritic arborizations (Fig. 2A,B). Terminal-like puncta were also present.

3.2.3. Insular cortex

On average, only half the animals receiving doses of either 20 mg/kg (one of four) or 40 mg/kg (three of four) contained lesions of the insular cortex. However, the total number of degenerating neurons was greater in this region than any other brain region (Table 2). Fluoro-Jade B-positive pyramidal and non-pyramidal cells were localized in mid layers of the posterior insular cortex. The continuum extended from the level of the hippocampal fornix caudally to the level of the hypothalamic arcuate nucleus, and extended from the rhinal fissure dorsally into ventral parietal cortex (Fig. 2E,F). Terminal degeneration was also conspicuous.

3.2.4. Ventral thalamus

Relatively large numbers of degenerating neurons were found throughout the entire extent of the ventromedial thalamus. Occasionally Fluoro-Jade B-positive neurons could be found extending into the ventral portion of the ventrolateral thalamus. Cells were medium size and of multipolar morphology (Fig. 2G). Labeled terminal-like puncta were also observed.

3.2.5. Intralaminar thalamus

A moderate number of degenerating neurons could be found within the intralaminar nucleus, being most numerous within the centrolateral division (Fig. 1). These

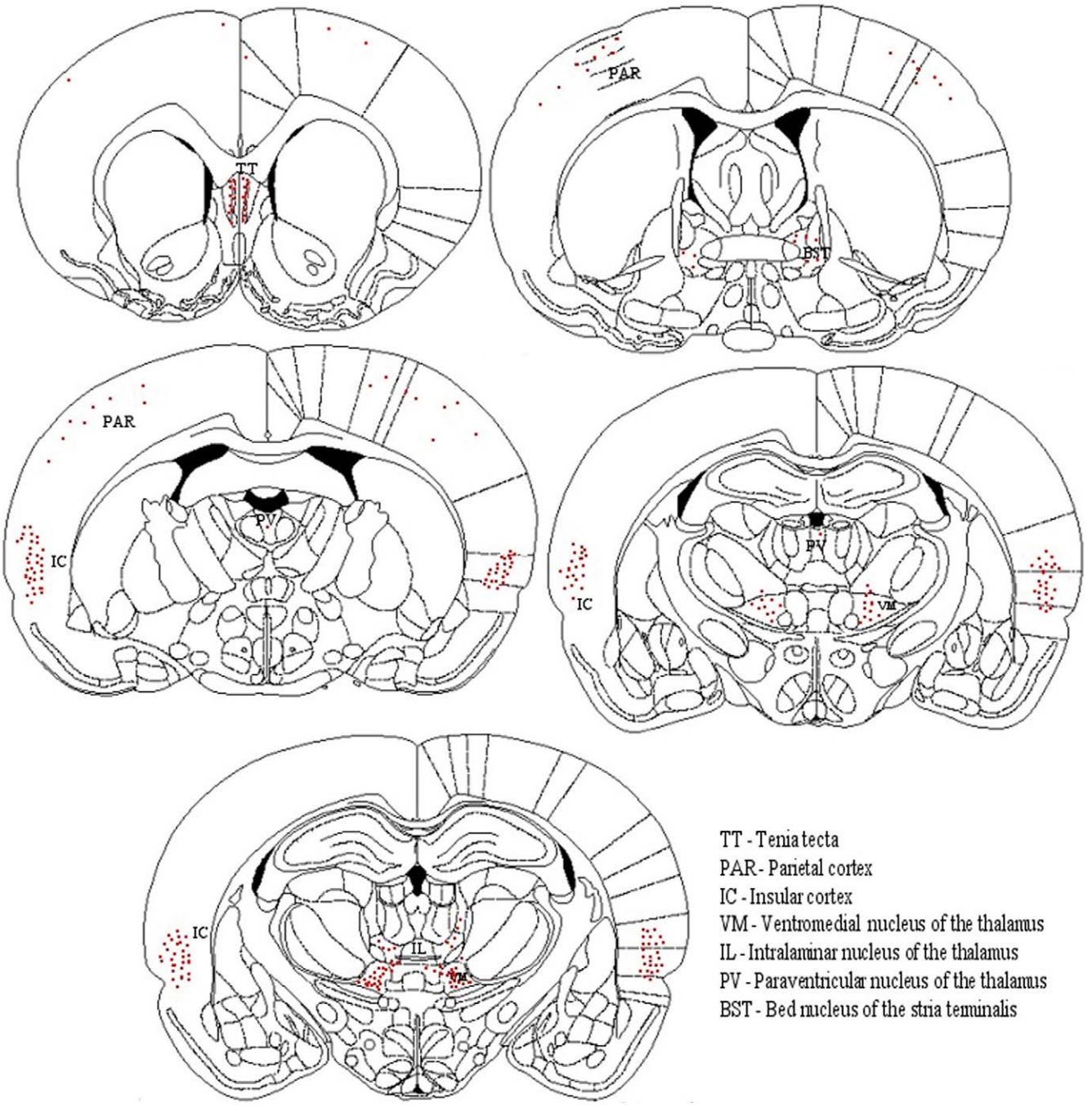
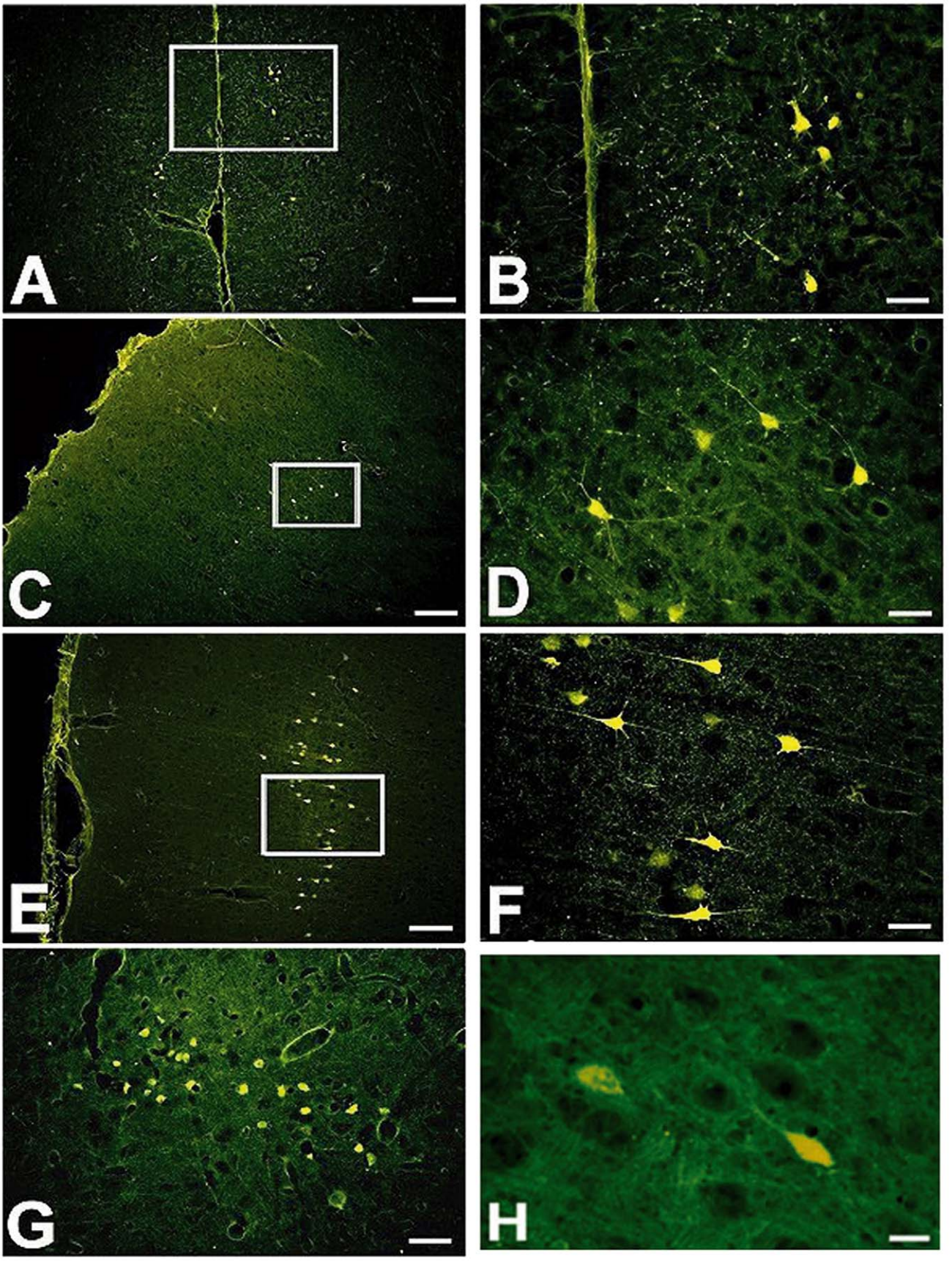


Fig. 1. The distribution of degenerating neurons from an individual given 20 mg/kg MDMA is plotted on templates of coronal sections taken from the rat brain atlas of Paxinos and Watson [12]. Temporal lobe structures reflect the nomenclature of Zilles and Wree [31]. Each red dot indicates one Fluoro-Jade-positive degenerating neuron. Abbreviations for brain regions containing Fluoro-Jade B-positive neurons are as follows: TT, tenia tecta; PAR, parietal cortex; IC, insular cortex; BST, bed nucleus of the stria terminalis; VM, ventromedial thalamic nuclei; PV, paraventricular thalamic nuclei; IL, intralaminar thalamic nuclei.

Fig. 2. Low- and high-magnification photomicrographs of brain regions from multiple individuals illustrates the regions most frequently found to contain Fluoro-Jade B-positive degenerating neurons following MDMA exposure: (A) Low magnification reveals degenerating neurons bilaterally in the tenia tecta on either side of midline. Blowup of insert is seen in (B). Scale bar=0.25 mm. (B) High-magnification view of the tenia tecta reveals Fluoro-Jade B-positive cells with granule cell-like morphology and medially oriented monopolar dendritic arborizations. Scale bar=40 μm. (C) Low-magnification view of Fluoro-Jade B-positive neurons in parietal cortex layers III and IV. This area is seen at high magnification in (D). Scale Bar=0.4 mm. (D) High magnification of labeled neurons within the parietal cortex reveals several types of morphology. Scale bar=40 μm. (E) Low-magnification view of the insular cortex reveals degeneration in mid-layers. Area in box is seen in (F). Scale bar=0.25 mm. (F) High-magnification view of insular cortex reveals both pyramidal and non-pyramidal Fluoro-Jade B-positive cells. Note conspicuous terminal-like puncta seen surrounding neurons. Scale bar=40 μm. (G) Medium size multipolar neurons of the ventromedial thalamus exhibit Fluoro-Jade B labeling. Scale bar=120 μm. (H) High-magnification view of two fusiform shaped cells of the intralaminar thalamus that are Fluoro-Jade B-positive. Dark regions correspond to unstained viable cell bodies. Scale Bar=25 μm.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Fluoro-Jade B-positive cells had a bipolar fusiform appearance (Fig. 2H).

3.2.6. Paraventricular thalamus

Relatively few labeled neurons were found in the thalamic paraventricular nucleus, which were generally small and rounded in appearance (not illustrated).

3.2.6.1. Bed nucleus of the stria terminalis (BST). Half of the animals (two of four) receiving the highest dose exhibited a modest number of medium-sized multipolar degenerating neurons in the BST at the level of the decussation of the anterior commissure (not illustrated).

4. Discussion

MDMA neurotoxicity is of particular relevance, due to its increasingly widespread illicit use since the mid 1980s [17]. Although some have reported a therapeutic role as an adjunct to psychotherapy [8,30], a number of other researchers have expressed concern over the neurotoxic potential of the compound (for reviews see Refs. [12,18]), especially regarding damage to serotonergic axon terminals. Although difficult to unequivocally prove terminal degeneration [12], there seems little question that the axons are severely damaged and functionally impaired, regardless of whether they have actually degenerated. This is reflected in the forebrain by the relatively long term loss of serotonin immunoreactivity [4,21,29], loss of axonal transport [18], reduction of 5-HT transporter density [2], and argyrophylia [4,10]. Although morphological changes have been reported for the serotonergic cells of origin in the dorsal raphe [1,17], there is no evidence that these cells actually degenerate. This does not, however, preclude the possibility of degeneration of other types of neurons. Indeed, as previously mentioned, there are two reports in the literature [4,10] of high doses of MDMA resulting in argyrophylic parietal cortex neurons.

Considering the major functional consequences of overt neuronal cell death, it is somewhat surprising that the phenomenon of MDMA-induced neuronal degeneration has received much less attention than its effects on serotonergic axon terminals, which may recover within a year of exposure [25]. One reason may be related to uncertainties and variabilities associated with suppressed silver methods. Both of the previous reports that mention MDMA-induced argyrophylia of parietal cortex neurons, also report silver stained neurons in control tissue. Another possible reason that the report of parietal cortex cell death did not attract more attention may be related to the unusually high doses (4×80 mg/kg i.p.) used in these early studies [4,10].

A related question is why was neuronal degeneration within forebrain regions other than the parietal cortex not detected previously? This may, in part, be attributed to the historical precedent set by the first study [5] to demonstrate that methamphetamine itself can cause neuronal degeneration of parietal cortex neurons, in addition to argyrophylic puncta within the striatum. Therefore many subsequent studies on the neurotoxicity of methamphetamine, as well as substituted methamphetamines such as MDMA, tended to focus on the aforementioned anatomical regions associated with the extrapyramidal motor system. There are other possible explanations as to why the more widespread pattern of neuronal degeneration seen in this study was not reported in earlier studies. Possible reasons include potential differences in animal ages, survival intervals, or housing conditions (e.g. ambient temperatures).

Although MDMA is generally characterized as a serotonin releaser [21,27], it has been shown to result in significant dopamine release as well [13]. Therefore, it is not surprising that the general lesion pattern observed for MDMA resembles a composite of the regions most vulnerable to dopamine releaser toxicity (e.g. methamphetamine [5,22]) plus those regions vulnerable to serotonin releaser neurotoxicity (e.g. D-fenfluramine [24]). It is interesting to note that most of the areas lesioned correlate well with regions receiving dense innervation by aromatic monoaminergic axons [28] including the tenia tecta, the paraventricular nucleus of the thalamus, the intralaminar thalamic nucleus, the parietal cortex and the insular/perirhinal cortex. However the story is more complex, as other brain regions with comparably high densities of aromatic monoaminergic fibers do not exhibit neuronal degeneration. Therefore, it is probable that the occurrence of neuronal degeneration depends on both the number of aromatic monoamine containing afferents, as well as the density and the specificity of post-synaptic receptors on the neurons fated to degenerate.

A common feature of aromatic monoamine releaser neurotoxicity is the requirement that the animal become hyperthermic for neurotoxicity to occur, as reflected by increased striatal GFAP immunoreactivity and decreased serotonin levels [11]. In the present study, only animals that achieved a body temperature in excess of 40.6 °C exhibited neuronal degeneration. Although neuronal degeneration was only seen in animals that became hyperthermic, previous studies [24] demonstrated that physically or chemically induced hyperthermia, in the absence of aromatic monoamine releasers, does not itself result in neuronal degeneration. MDMA-induced hyperthermia is not limited to rodents and is seen in primates as well. This suggests a certain level of hyperthermia will be predictive of neuronal degeneration in humans as well. It is of interest to note that humans with clinical symptoms of MDMA neurotoxicity had body temperatures between 40 °C and 43.3 °C [27]. This raises the concern that humans may receive a dose capable of causing irreversible neuronal degeneration. In the rat, we found that select individuals were susceptible to neuronal degeneration at a dose of 10 mg/kg, while all rats given a dose of 20 mg/kg, or more, exhibited neuronal degeneration. Thus, neuronal degeneration was seen in the rat at doses 5–10 times higher than a

'typical' human dose of 2 mg/kg [18]. Since humans have a much greater body weight to surface area ratio than the rat, one would predict that they will not dissipate heat as efficiently and therefore be more susceptible to drug-induced hyperthermia. This assumption is supported by a number of studies [7,11,17,19]. The fact that the human dosage of MDMA is not standardized and that some users frequently take multiple doses over short intervals [16,19,27] raises the concern that humans, who take doses of MDMA producing sufficient hyperthermia, will suffer irreversible loss of neurons in the aforementioned forebrain regions.

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