

# Decrease in REM latency and changes in sleep quality parallel serotonergic damage and recovery after MDMA: a longitudinal study over 180 days



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## Abstract

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. Since there is evidence that serotonin regulates sleep, we have compared serotonergic markers parallel with detailed analysis of sleep patterns at three time-points within 180 d after a single dose of 15 mg/kg MDMA in male Dark Agouti rats. At 7 d and 21 d after MDMA treatment, significant (30–40%), widespread reductions in serotonin transporter (5-HTT) density were detected in the cerebral cortex, hippocampus, most parts of the hypothalamus, and some of the brainstem nuclei. With the exception of the hippocampus, general recovery was observed in the brain 180 d after treatment. Transient increases followed by decreases were detected in 5-HTT mRNA expression of dorsal and median raphe nuclei at 7 d and 21 d after the treatment. Significant reductions in rapid eye movement (REM) sleep latency, increases in delta power spectra in non-rapid eye movement sleep and increased fragmentation of sleep were also detected, but all these alterations disappeared by the 180th day. The present data provide evidence for long-term, albeit, except for the hippocampus, transient changes in the terminal and cellular regions of the serotonergic system after this drug. Reduced REM latency and increased sleep fragmentation are the most characteristic alterations of sleep consistently described in depression using EEG sleep polygraphy.

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## Introduction

'Ecstasy' [3,4-methylenedioxymethamphetamine (MDMA)], an amphetamine analogue used recreationally by humans, has been found to damage

brain serotonin (5-HT) neurons in experimental animals, and reportedly, possibly also in human MDMA users (McCann et al., 1998, 2005; Semple et al., 1999).

The acute effects of MDMA involve several neurotransmitters, e.g. monoamines and acetylcholine, but the long-term effects are selective for the serotonergic system in the brain (Colado et al., 1999; Green et al., 2003) and thus the serotonergic system might serve as a model for selective, partial lesion of this system. In animal models, marked reductions were observed in

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the concentration of 5-HT, 5-hydroxyindolacetic acid and serotonin transporter (5-HTT) density (Battaglia et al., 1987; Colado et al., 1993; Green et al., 2003; Sabol et al., 1996). There is also 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. Based mainly on these findings, it has been proposed that MDMA is more toxic to 5-HT projection neurons in the dorsal raphe nucleus (DRN) than those in the median raphe nucleus (MRN) (Molliver et al., 1989; O'Hearn et al., 1988). However, expression of 5-HTT mRNA in the raphe nuclei has not been examined; consequently, direct comparisons of responses of dorsal and other raphe nuclei have not been measured.

5-HT plays a key role in the regulation of sleep, especially rapid eye movement (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). 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 involved massive depletions of 5-HT with sleep studies generally performed shortly after lesioning (Allen et al., 1993; Koella et al., 1968; Touret et al., 1991).

The present study was designed to characterize MDMA-induced damage and recovery of the serotonergic system including both cellular and terminal events. Furthermore, we hypothesized that long-lasting partial damage and recovery of this system will be manifested in changes of sleep parameters. For these purposes we investigated parallel changes in 5-HTT mRNA expression in two raphe nuclei, 5-HTT fibre densities in several brain areas, and 16 functional measures of sleep in response to MDMA administration at three time-points within 180 d.

## Method

### *Animals*

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Principles of Laboratory Animal Care (NIH Publications No. 85-23, revised 1985), as well as specific national laws (the Hungarian

Governmental Regulation about Animal Studies, 31 December 1998). Permission was also obtained from the local ethical committees. Male Dark Agouti rats aged 7 wk (Harlan, Olac Ltd, Shaw's Farm, Blackthorn, Bicester, Oxon, UK) were used in the experiments. The animals (four per cage) were kept under controlled environmental conditions (temperature  $21 \pm 1$  °C, and a 12 h light-dark cycle, lights on at 06:00 hours). Standard food and drinking water were freely available.

### *Drug administration and treatment protocol*

(±)3,4-Methylenedioxymethamphetamine hydrochloride (MDMA, certified reference compound, purity >99.5%) was kindly provided by Professor Istvan Hermecz (Sanofi-Synthelabo-Chinoin, Budapest, Hungary). The drug was dissolved in 0.9% NaCl at a dose equivalent to 15 mg/kg free base and was injected intraperitoneally in a volume of 1 ml/kg. Control animals received an injection of 0.9% NaCl in a volume of 1 ml/kg. To measure the time-course of 5-HTT mRNA expression after MDMA treatment, in-situ hybridization histochemistry was performed 3, 7, 21 and 180 d after drug treatment ( $n=48$ , six animals in each control and treated group at the four time-points). In parallel, the density of serotonergic fibres was assessed in separate group of rats using 5-HTT immunohistochemistry ( $n=5-6$  per group). Separate groups of animals were used for vigilance studies ( $n=6-9$  per group).

### *In-situ hybridization histochemistry*

In-situ hybridization was performed as previously described (Hansson et al., 1998; Kovacs et al., 2007). After hybridization, sections were exposed to a BAS-MS 2340 Imaging Plate (FujiPhotoFilm Co. Ltd, Tokyo, Japan) for 6–13 d, depending on the intensity of the hybridization signal. The hybridization signals were quantified with ImageJ 1.32j imaging software (Wayne Rasband, NIH, Bethesda, MD, USA). For quantification, we averaged 9–9 sections for the DRN (between  $-9.30$  and  $-7.30$  mm from the level of bregma) and 8–8 for the MRN (between  $-8.30$  and  $-7.30$  mm from the level of bregma) per animal. 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 DRN or MRN were measured on the film autoradiograph, and also over a similar size of the surrounding area that did not contain serotonergic cells. Differences measured in grey density were used for evaluation and statistical analysis.

To obtain more precise measures for the effects of MDMA, 5-HTT mRNA expression of individual cells was counted. The sections were coated with nuclear track emulsion (NTB-2, Kodak, Rochester, NY, USA) for 4 wk at 4 °C. For each experimental animal, 4–4 sections were analysed, and the number of silver grains was measured over serotonergic cell bodies (25 or 10 cells in the DRN and MRN, in each section, respectively). The threshold was adjusted to the background signal level, the area in question was selected and the mean grey density was automatically calculated with respect to the surface area of the selected section. The mean grey density values reflect the mRNA expression of the cell.

### *Immunohistochemistry*

The animals were deeply anaesthetized with nembutal, and perfused transcardially with Zamboni fixative solution. Brains were removed and post-fixed overnight at 4 °C in fixing solution. Free-floating 40- $\mu$ m-thick coronal sections were cut using a freezing microtome. The presence of 5-HTT was detected using a 1:3000 dilution of a rabbit polyclonal anti-5-HTT antibody (Oncogene, San Diego, CA, USA). Immunostaining was developed with a peroxidase/DAB kit (EnVision™, Dako, Glostrup, Denmark).

Monochrome digital images were made using similar light microscopic settings (Olympus BX51, objective, aperture, exposure time) at  $\times 40$  magnification. Four to six representative, non-overlapping photographs were taken in each anatomical region: hippocampus (dentate gyrus, CA1, CA2, CA3) (3.60–3.20 mm caudal to bregma), hypothalamus (posteroventral preoptic areas (0.30–0.40 mm), lateral hypothalamic area (3.60–3.20 mm), posterior hypothalamic nucleus (3.60–4.30 mm), paraventricular nucleus (1.50–2.10 mm), suprachiasmatic nucleus (SCN) (0.90–1.40 mm), tuberomammillary nucleus (3.80–4.00 mm), cerebral cortex layers I–II [somatosensory and somatomotor cortex (3.30–2.30 mm)], periaqueductal grey [ventrolateral and dorsomedial part (7.80–8.30 mm)], peripeduncular tegmental nucleus (7.80–8.30 mm) and medial pontine reticular formation (8.80–7.80 mm).

Quantitative analysis of serotonergic fibre density was performed using analySIS image software (Soft Imaging System GmbH, Lakewood, CO, USA). The positively stained fibres were distinguished from the background by means of density thresholding, and the area occupied by immunostained fibres was determined and recorded as a percentage of the total image area.

### *Vigilance studies*

#### *Surgery*

Animals were chronically equipped with electroencephalogram (EEG) and electromyogram (EMG) electrodes, as described previously (Filakovszky et al., 2001; Kantor et al., 2002). Surgery was performed under halothane (2%) anaesthesia (Fluotec 3) using Kopf stereotaxic instrument. After a 10-d 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 intraperitoneal injections of physiological saline daily for 5 d before the experiments.

#### *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., 2002). The vigilance states were scored visually for 4-s periods for the first 2 h after light onset using conventional criteria (Kantor et al., 2002, 2004). The polygraphic recordings were classified by sleep analysis software for the 24-h recordings (SleepSign for Animal, Kissei Comtec America Inc.). EEG power spectra were computed for consecutive 4-s epochs at the frequency range of 0.25–30 Hz (fast Fourier transformation routine, Hanning window; frequency resolution, 0.25 Hz) (Kantor et al., 2002, 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 sleep continuity. The REM sleep parameters included REM latency [the time elapsed from sleep onset until the first seven 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 occurred) 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-s epochs, or a cumulated total of 240 s 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 was calculated from light onset), SWS-1 and SWS-2 latencies (the time

between light onset and the first cumulated total of a minimum 28 s 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 increased by a minimum of 50% compared to the average in the passive phase) and the passive wake before activity onset (duration of passive wake during the hour preceding the activity onset). Sleep continuity was defined as the sum of the number of awakenings (either active wake or passive wake) that disconnected any sleep periods [SWS-1, SWS-2, intermediate stage of sleep (IS), or REM] during the light period, the passive phase (0–12 h).

### Statistical methods

Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis. Morphological data and 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 analysed using one- or two-way (treatment and time) analysis of variance (ANOVA) and Newman–Keuls test for post-hoc comparisons. REM sleep in the first hour and NREM sleep in the first and second hour were also evaluated by multivariate analysis of variance (MANOVA) for repeated measures. In the case of time of REM maximum and activity onset the Mann–Whitney *U* test was performed. Log-transformed values were used for the statistical analysis of the EEG power spectra data.

## Results

### 5-HTT mRNA expressions in the dorsal and median raphe nuclei

#### Dorsal raphe nucleus

The changes in 5-HTT mRNA expression density were quantified by film autoradiography at each time-point studied (3, 7, 21 and 180 d after MDMA treatment) (Figure 1a, b). Alterations in 5-HTT mRNA expression were further analysed on slides dipped into autoradiographic emulsion (Figure 2a). The levels of 5-HTT mRNA in the DRN between groups are shown in the autoradiographs in Figure 1(a). In general, significant treatment  $\times$  time interaction ( $F_{3,40}=6.369$ ,  $p=0.001$ ) was found for this parameter. A significant

increase was observed 7 d after drug administration (+24%,  $p<0.01$ ) (Figure 1a).

To obtain more sensitive measures for the effects of MDMA, 5-HTT mRNA expression of individual cells was counted at 7 and 21 d after drug or saline treatment (Figure 3). We noted significantly elevated 5-HTT mRNA expression at 7 d (+53%,  $p<0.05$ ) (Figures 2a, 4a, c) and significantly reduced 5-HTT mRNA expression at 21 d after MDMA treatment (–22%,  $p<0.05$ ) (Figures 2a, 4e, g).

MDMA did not affect the levels of 5-HTT mRNA in the DRN compared to the control group at 3 and 180 d after drug exposure (Figure 1a, b).

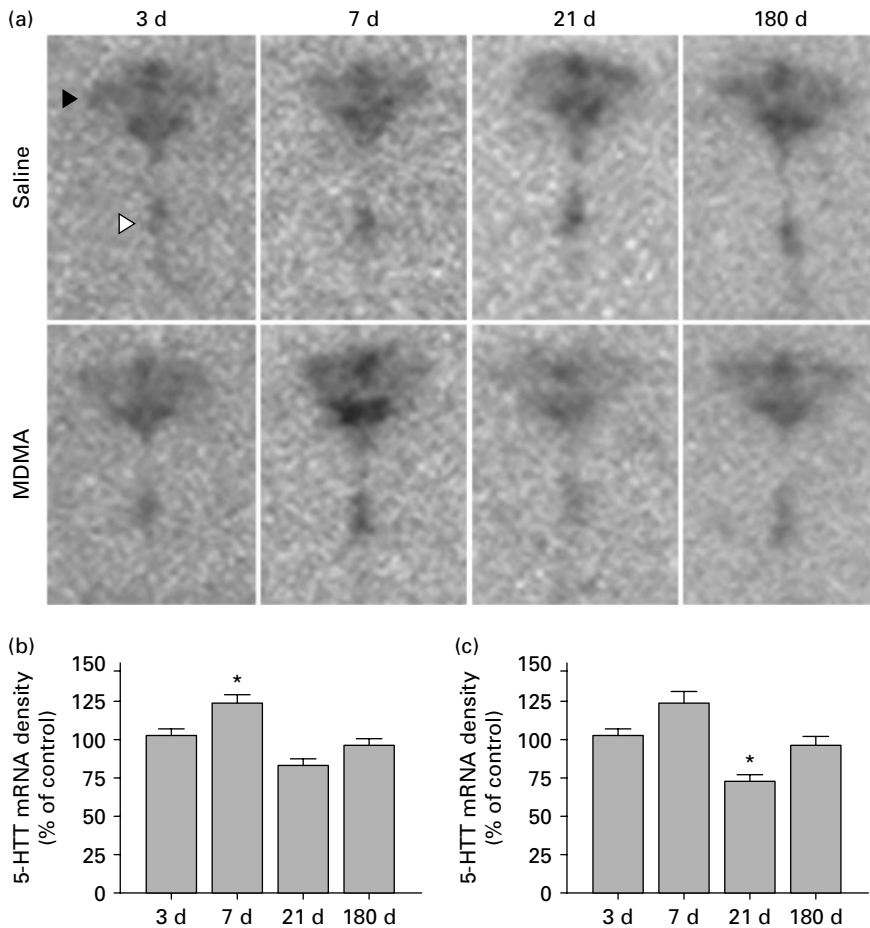
#### Median raphe nucleus

In the MRN, 5-HTT mRNA density was quantified by film autoradiography at each survival time (3, 7, 21 and 180 d after MDMA treatment) (Figure 1a, c), and the changes in 5-HTT mRNA expression were further compared by analysis of the signal from the slides dipped into autoradiographic emulsion, similar to the measurements on the DRN (Figure 2b). In general, significant treatment  $\times$  time interaction ( $F_{3,39}=4.790$ ,  $p=0.006$ ) was found in the MRN. As shown in Figure 1(c), 5-HTT mRNA expression significantly decreased 21 d (–27%,  $p<0.05$ ) after drug exposure in the MRN. To support these results by a more sensitive method, 5-HTT mRNA expression of individual cells was counted 7 and 21 d after MDMA administration. We found significant increase in the levels of 5-HTT mRNA at 7 d (+43%,  $p<0.05$ ) (Figures 2b, 4b, d) and significant decrease at 21 d after MDMA treatment (–18%,  $p<0.05$ ) (Figure 2b, 4f, h). No differences between controls and treated animals were observed at 3 and 180 d after MDMA administration (Figure 1a, c). Qualitatively, all of these effects were similar to the changes observed in the DRN.

### Density of 5-HTT immunoreactive fibres in the brain

#### Cerebral cortex

Treatment with MDMA significantly reduced 5-HTT fibre density at 7 and 21 d in the somatosensory (treatment effect:  $F_{1,28}=37.791$ ,  $p<0.0001$ ; Figure 5a–d) and somatomotor cortex (treatment effect:  $F_{1,28}=74.456$ ,  $p<0.0001$ ; Table 1). Significant treatment  $\times$  time interaction was observed in the somatomotor cortex ( $F_{2,56}=6.665$ ,  $p=0.0025$ ). The percentage changes in the somatosensory cortex and somatomotor cortex were similar: –31%, –33%, –15%, and –36%, –32%, –14%, at 7, 21 and 180 d after treatment, respectively. The reduction in 5-HTT fibre



**Figure 1.** Expression of 5-HTT mRNA in control (saline) rats, and in rats at 3, 7, 21 and 180 d after treatment with a single dose of MDMA (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 (b) the dorsal raphe nucleus and (c) the median raphe nucleus in rats exposed to MDMA 3, 7, 21, 90 and 180 d earlier. 5-HTT mRNA is expressed as a relative ratio compared to controls (%). Data are mean  $\pm$  S.E.M. ( $n=6$ ). \* Significantly different compared to the saline group ( $p<0.05$ ).

density was not significant 180 d after drug exposure (Figure 5e).

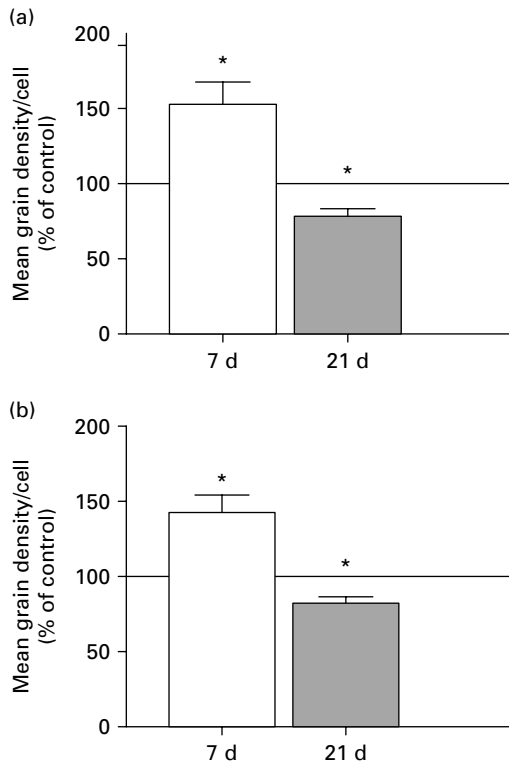
#### Hippocampus

In the hippocampus, we found an overall decrease in the 5-HTT fibre density, which was present from day 7 up to day 180 after treatment (Table 1). We observed significant decreases in all hippocampal areas examined (CA1 treatment effect:  $F_{1,28}=27.012$ ,  $p<0.0001$ ; CA2 treatment effect:  $F_{1,28}=57.202$ ,  $p<0.0001$ ; CA3 treatment effect:  $F_{1,28}=48.173$ ,  $p<0.0001$ ), except for the dentate gyrus. Post-hoc tests indicated significantly reduced 5-HTT fibre density in the CA1 at all time-points examined ( $-26\%$ ,  $-37\%$ ,  $-36\%$ ) (Figure 5f–j), CA2 ( $-35\%$ ,  $-41\%$ ,  $-32\%$ ) and CA3 ( $-33\%$ ,  $-46\%$ ,  $-31\%$ ) at 7, 21 and 180 d after

treatment, respectively. A reduction in 5-HTT fibre density was also noted in the dentate gyrus but this change was not significant ( $-24\%$ ,  $-35\%$ ,  $-19\%$ , at 7, 21 and 180 d after treatment, respectively).

#### Hypothalamus

The effects of MDMA on 5-HTT fibre density were different in different hypothalamic nuclei and areas (Table 1). Reduced 5-HTT fibre density was found in the posterior hypothalamic nucleus (treatment effect:  $F_{1,27}=32.256$ ,  $p<0.0001$ ), SCN (treatment effect:  $F_{1,27}=30.423$ ,  $p<0.0001$ ) and tuberomammillary nucleus (treatment effect:  $F_{1,27}=35.121$ ,  $p<0.0001$ ); the lateral hypothalamic area; posteroventral preoptic areas and the paraventricular nucleus were resistant to MDMA at all time-points measured. Alterations in 5-HTT

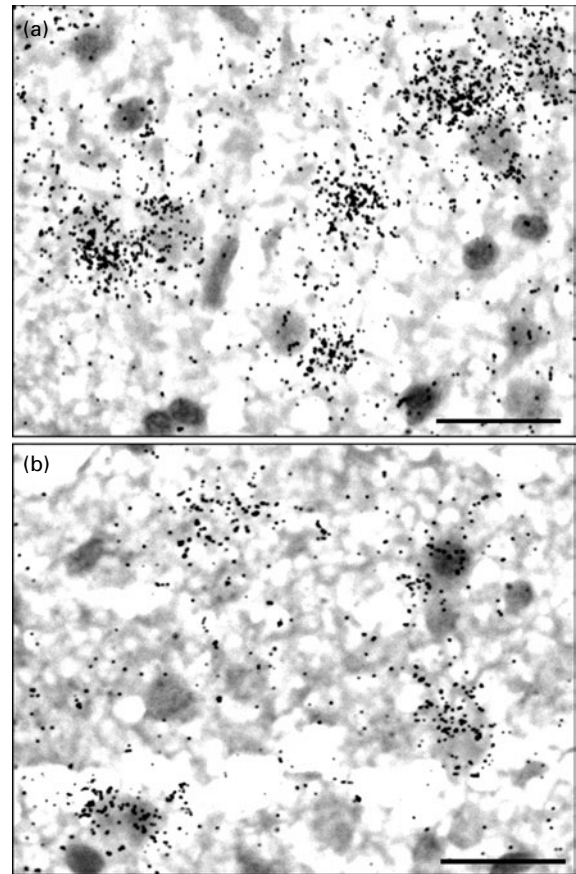


**Figure 2.** Quantification of 5-HTT mRNA expression by measuring grain density in individual cells of (a) the dorsal raphe nucleus and (b) the median raphe nucleus at 7 and 21 d after MDMA treatment. Values represent mean grain density/cell ratio relative to controls. Data are mean  $\pm$  S.E.M. ( $n=6$ ). \* Significantly different compared to the control (saline) group ( $p < 0.05$ ).

fibre density were as follows: lateral hypothalamic area (–12%, –19%, –8%); posterior hypothalamic nucleus (–22%, –38%, –30%); SCN (–30%, –35%, –17%); tuberomammillary nucleus (–19%, –35%, –23%); posteroventral preoptic areas (–11%, –17%, –2%); paraventricular nucleus (–2%, –6%, –3%), at 7, 21 and 180 d after MDMA, respectively.

#### Brainstem

In general, changes in the brainstem were smaller, and significant alterations were observed only in two regions: the ventrolateral cell groups (treatment effect:  $F_{1,27}=16.452$ ,  $p=0.0004$ ) and dorsomedial cell groups (treatment effect:  $F_{1,27}=15.212$ ,  $p=0.0006$ ) of the periaqueductal grey matter in the midbrain, but only at 21 d (Table 1). The effects of MDMA on 5-HTT fibre density in the peripeduncular tegmental nucleus and the medial part of the pontine reticular formation were not significant at any time-point studied. Post-hoc comparison revealed the following changes in 5-HTT



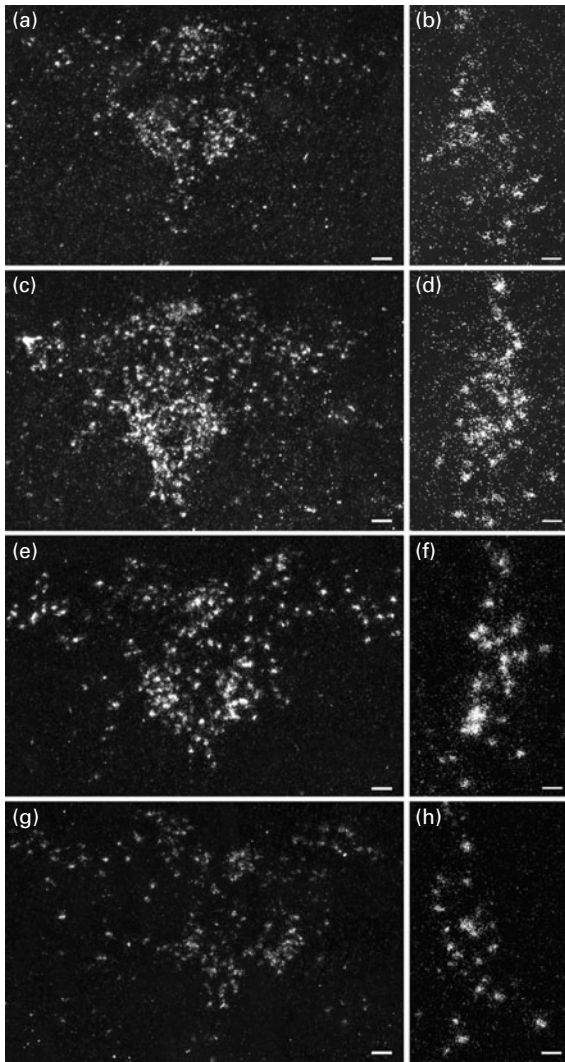
**Figure 3.** Bright-field image showing the cellular localization of 5-HTT mRNA in the dorsal raphe nucleus in (a) control rats, and (b) in rats 21 d after treatment with a single dose of MDMA. Panel (b) shows decreased grain density. Scale bars, 25  $\mu$ m.

fibre density: periaqueductal central grey ventrolateral part (–15%, –30%, –15%); periaqueductal central grey dorsomedial part (–17%, –36%, –16%); peripeduncular tegmental nucleus (–7%, –12%, –7%); medial pontine reticular formation (–3%, –14%, –5%), at 7, 21 and 180 d after treatment, respectively.

#### Effects of MDMA on sleep parameters

##### REM sleep

Complete 24 h recordings of REM sleep are shown in Figure 6(a–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 2). Significant decreases in REM latency were detected at 7 and 21 d after



**Figure 4.** Dark-field images show 5-HTT mRNA expression in the dorsal raphe nucleus (a, c, e, g) and median raphe nucleus (b, d, f, h) in control (a, b, e, f) rats and in rats treated with MDMA 7 d (c, d) and 21 d (g, h) earlier. 5-HTT mRNA revealed by in-situ hybridization with a  $^{35}\text{S}$ -labelled ribonucleotide probe. Increased 5-HTT mRNA expression at 7 d following a single dose of MDMA (c, d), compared to controls (a, b). A marked decrease in 5-HTT mRNA is shown in panels (g) and (h) at 21 d following a single dose of MDMA compared to controls (e, f). Scale bars: (a, c, e, g), 100  $\mu\text{m}$ ; (b, d, f, h), 50  $\mu\text{m}$ .

MDMA administration (treatment effect:  $F_{1,36} = 22.880$ ,  $p < 0.0001$ ). Changes in REM latency were  $-55\%$ ,  $-53\%$ , and  $-29\%$ , at 7, 21 and 180 d after MDMA, respectively. In parallel, increased duration of REM ( $+245\%$ ) was found in the first hour of the passive phase 21 d after MDMA (treatment effect:  $F_{1,46} = 7.689$ ,  $p = 0.0079$ ). Duration of REM sleep analysed during the whole light period ( $-15\%$ ,  $-34\%$ ,  $-15\%$ , at 7, 21

and 180 d after treatment, respectively) was not significantly affected by MDMA treatment. The time of actual maximum of REM sleep was not significantly altered ( $+64\%$ ,  $+30\%$ ,  $-9\%$ , at 7, 21 and 180 d after MDMA, respectively). At 7 d, 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 6a).

#### NREM sleep

Nine parameters (NREM latency, NREM in first and second hour, SWS-1 and SWS-2 latency, delta power SWS-1 and SWS-2 in a 2-h recording period beginning at light onset 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 2; Figure 7). Differences in NREM latency were not significant at any time-point ( $-13\%$ ,  $-15\%$ ,  $0\%$ , at 7, 21 and 180 d after treatment, respectively). Neither SWS-1 ( $+8\%$ ,  $-51\%$ ,  $3\%$ , at 7, 21 and 180 d after treatment, respectively) nor SWS-2 latencies ( $-2\%$ ,  $-6\%$ ,  $1\%$ , at 7, 21 and 180 d 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 d after treatment, respectively) and second hour ( $-10\%$ ,  $-4\%$ ,  $0\%$ , at 7, 21 and 180 d 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 d after MDMA treatment (treatment  $\times$  time interaction:  $F_{2,40} = 4.847$ ,  $p = 0.013$ ;  $+8\%$ ,  $-51\%$ ,  $-3\%$ , at 7, 21 and 180 d after treatment, respectively). Delta power measured either in SWS-1 or in SWS-2 was increased by MDMA at both 7 and 21 d after treatment (Figure 7a, b, d, e). Changes in the lower frequencies (1 Hz 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 d (Figure 7a, e). 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 animals.

#### Wake parameters around activity onset

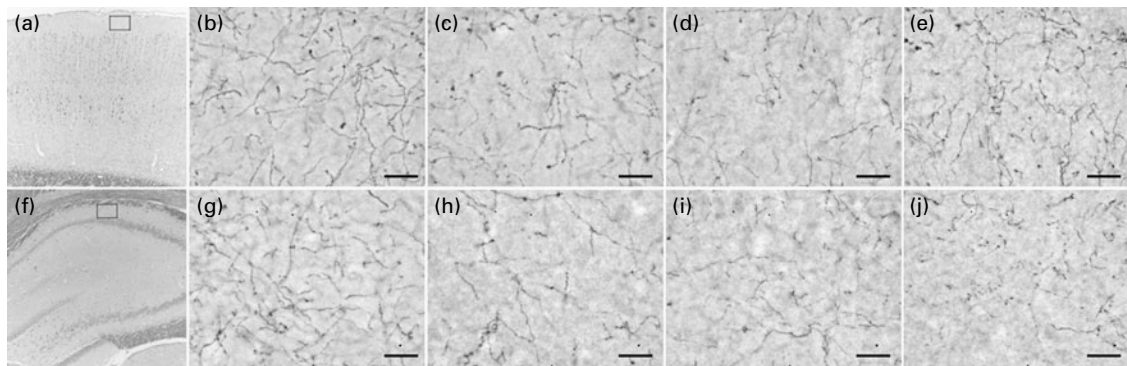
Activity onset preceding the active phase was determined for each animal, in each experimental group. In general, activity was increased by 50% compared to the preceding 8-h resting period 2–3 h before lights off. No significant MDMA effect was found for this

**Table 1.** 5-HTT fibre density at 7, 21 and 180 d after MDMA (15 mg/kg) treatment in brain areas of Dark Agouti rats

	7 d		21 d		180 d	
	Saline	MDMA	Saline	MDMA	Saline	MDMA
<b>Cerebral cortex</b>						
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
<b>Hippocampus</b>						
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
<b>Hypothalamus</b>						
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
Tuberomammillary 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*
<b>Brainstem</b>						
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
<b>Periaqueductal central grey</b>						
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

Data are presented as mean ± S.E.M. of 5–6 animals per group.

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



**Figure 5.** 5-HTT immunoreactive axons in the somatosensory cortex and in the hippocampal CA1 region. Representative photomicrographs illustrating the areas where the densitometry was applied [open rectangles in panels (a) and (f)], and 5-HTT fibre density in control (b, g) and MDMA-treated rats at 7 d (c, h), 21 d (d, i) and 180 d (e, j) after treatment. Decreases in 5-HTT fibre density can be observed in panels (c, d, h, i). Scale bars, 20  $\mu$ m.

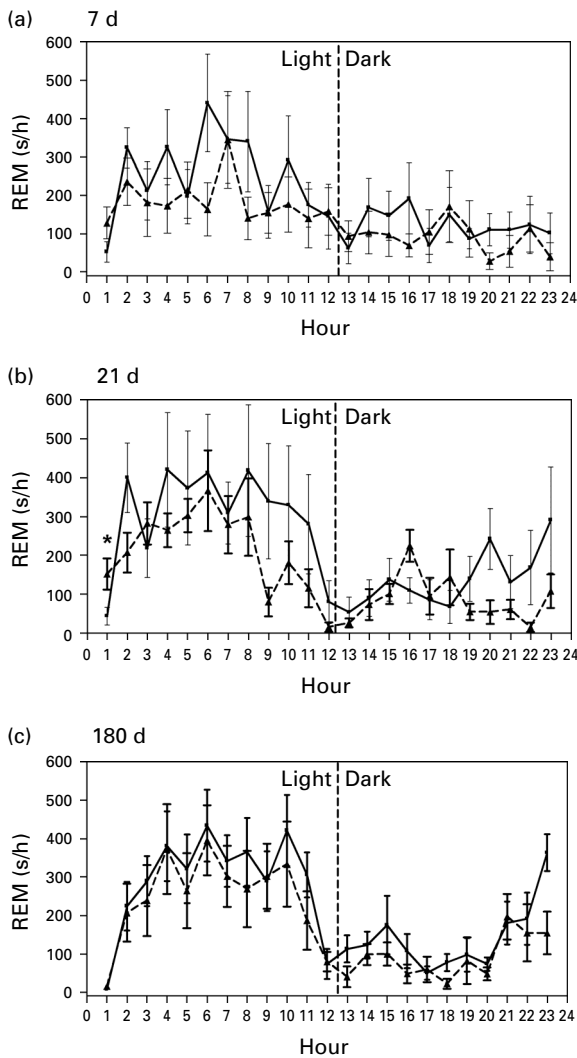
parameter (+17%, 0%, 0%, at 7, 21 and 180 d after treatment, respectively). Duration of passive wake during the hour preceding the activity alteration was also calculated. Changes in the duration of passive wake before activity onset were +5%, -28%, -21%, at 7, 21 and 180 d after treatment, respectively. None of

these parameters were significantly altered by MDMA treatment (Table 2).

#### *Sleep continuity*

The number of wake periods during the 12-h passive phase was used to characterize sleep continuity.





**Figure 6.** Twenty-four-hour recordings of rapid eye movement (REM) sleep in MDMA-treated ( $- \blacktriangle -$ ) and saline-treated ( $- \blacksquare -$ ) animals at 7 d (a), 21 d (b) and 180 d (c) after treatment. Each point represents mean values  $\pm$  S.E.M. ( $n = 6-8$ ) of the time spent in REM sleep within each hour. \* Significantly different compared to the saline group ( $p < 0.05$ ).

MDMA caused significant increase in this parameter but only at day 7 (+83%, +12%, -32%, at 7, 21 and 180 d after treatment, respectively) (Table 2). The treatment  $\times$  time interaction ( $F_{2,41} = 7.306$ ,  $p = 0.0019$ ) was significant.

## Discussion

Parallel measurements of 5-HTT mRNA expression in the two raphe nuclei, 5-HTT fibre density in several brain areas, and more than a dozen functional

measures of sleep at three time-points within 180 d 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.

Acute and subacute effects of MDMA on sleep have been previously described by our group (Balogh et al., 2004). Here we concentrated on chronic and persistent morphological and functional effects of the drug.

In the present study, 5-HTT mRNA expression was measured in the two largest groups of ascending projecting serotonergic neurons, the DRN and the MRN. A transient increase followed by a transient decrease and a complete recovery were found during the 180-d period in both raphe nuclei studied. The effects of MDMA on 5-HTT mRNA expression in DRN and MRN did not differ in time or extent. The up-regulation of 5-HTT mRNA expressions 7 d after MDMA treatment in both 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 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 d. Evidence for partial recovery was found in most affected brain regions, except for the hippocampus, by 180 d.

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 DRN, while thicker fibres are derived from the MRN (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, later neuro-anatomical and biochemical studies suggest that DRN 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 MRN origin are also affected by MDMA. The origin of serotonergic projections to the SCN is almost exclusively the MRN (Glass et al., 2003; Meyer-Bernstein et al., 1999), and

**Table 2.** Sleep parameters at 7, 21 and 180 d after MDMA (15 mg/kg) treatment

Sleep parameter	7 d		21 d		180 d	
	Saline	MDMA	Saline	MDMA	Saline	MDMA
<b>REM sleep</b>						
REM latency (s)	3745 ± 446	1676 ± 249*	2937 ± 364	1383 ± 160*	3511 ± 647	2485 ± 451
REM in 1st hour (s)	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 (h)	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 (s)	2485 ± 684	2107 ± 429	3742 ± 1277	2453 ± 458	3465 ± 660	2959 ± 766
<b>NREM sleep</b>						
NREM latency (s)	1798 ± 367	1558 ± 269	1986 ± 412	1687 ± 515	2201 ± 394	2195 ± 429
NREM in first hour (s)	1107 ± 217	1414 ± 167	891 ± 127	1410 ± 248	663 ± 144	801 ± 179
NREM in second hour (s)	2230 ± 196	1998 ± 113	2280 ± 188	2188 ± 157	2136 ± 95	2133 ± 122
SWS-1 latency (s)	1585 ± 395	1717 ± 250	1528 ± 458	746 ± 225	2008 ± 254	2064 ± 315
SWS-1 delta power ( $\mu\text{V}^2/\text{Hz}$ )	3218 ± 254	4874 ± 588*	3066 ± 139	3461 ± 87	2602 ± 205	2527 ± 456
Sum of SWS-1 in the light period (s)	7259 ± 751	6910 ± 1139	8222 ± 770	4444 ± 287*	10751 ± 1076	12473 ± 1071
SWS-2 latency (s)	1993 ± 465	1962 ± 228	2208 ± 425	2069 ± 461	3578 ± 358	3610 ± 302
SWS-2 delta power ( $\mu\text{V}^2/\text{Hz}$ )	8813 ± 1230	12005 ± 1394	7255 ± 632	11478 ± 1108*	4727 ± 321	5313 ± 723
Sum of SWS-2 in the light period (s)	16779 ± 1400	18502 ± 1523	16336 ± 996	20180 ± 591	10776 ± 872	9738 ± 1510
<b>Wake parameters around activity onset</b>						
Activity onset (h)	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 (s)	501.5 ± 87.5	528.6 ± 128.2	604.0 ± 82.8	435.5 ± 77.5	611.5 ± 141.1	483.5 ± 111.1
<b>Sleep continuity</b>						
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

REM, Rapid eye movement; NREM, non-rapid eye movement; SWS-1, light slow-wave sleep; SWS-2, deep slow-wave sleep.

Data are presented mean  $\pm$  S.E.M. of 7–9 animals per group.

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

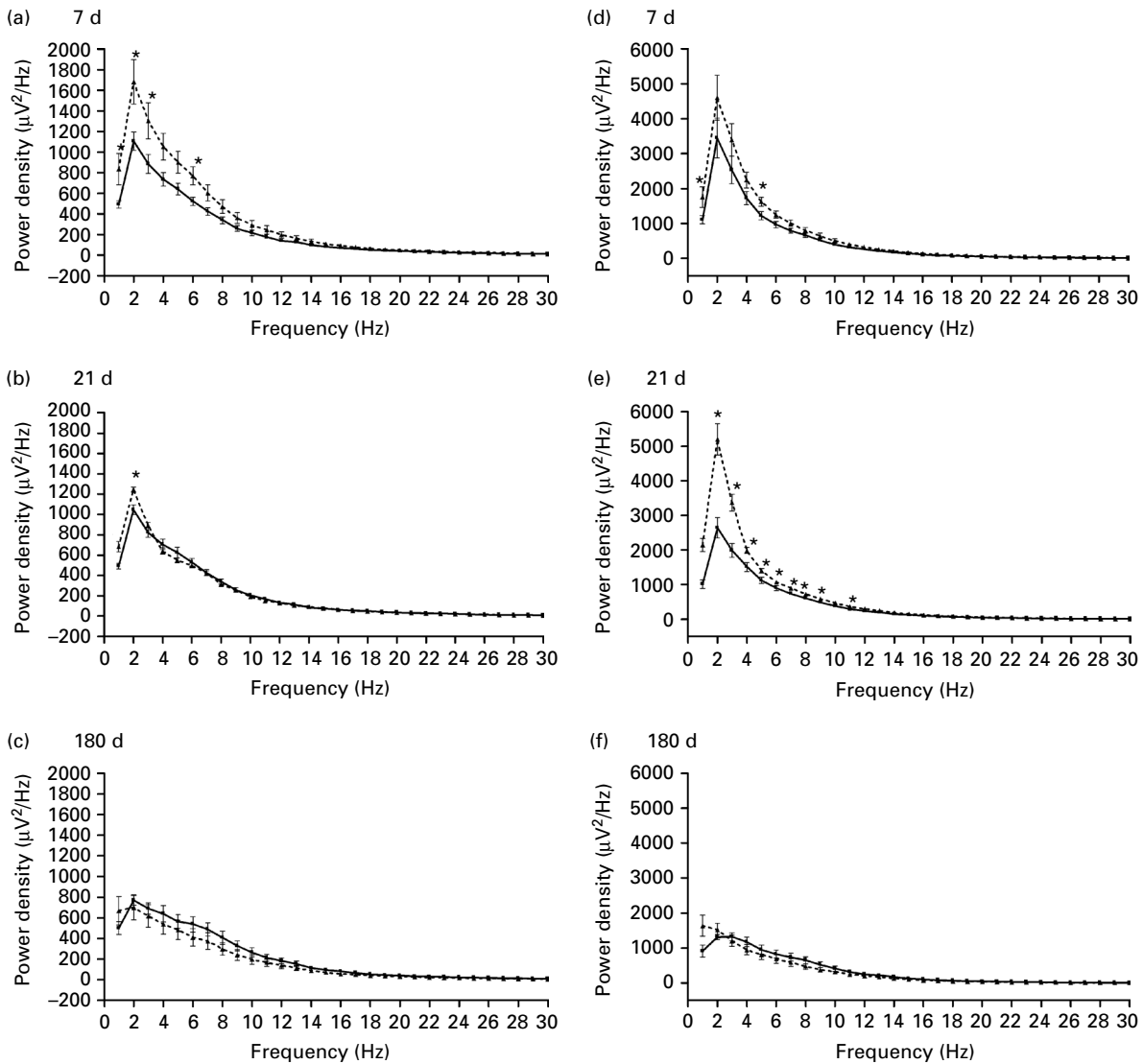
MRN median raphe fibres project heavily throughout the entire hippocampus (Azmitia and Segal, 1978; Conrad et al., 1974; Imai et al., 1986; Vertes and Martin, 1988). The SCN and the three CA areas of the hippocampus were strongly affected by MDMA in our experiment. Furthermore, changes in 5-HTT mRNA expression in the DRN and MRN were similar in the present study. These data support the view that axons of both DRN and MRN origin are temporarily damaged by the drug.

Recovery of serotonergic neurons was observed in animals treated with a single dose of MDMA 180 d previously in most areas examined except for the hippocampus. This observation supports previous results that the rate and degree of recovery is region dependent (Sabot et al., 1996). Scanzello et al. (1993) found that the hippocampus is the only brain region where none of the presynaptic 5-HT neuronal markers recovered 1 yr after MDMA administration.

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). Among the consequences of these findings might be long-term changes in memory functions and/or anxiety. Indeed, human studies most consistently point to cognitive and memory disturbances in current and previous ecstasy users (Morgan, 2000; Parrott, 2001).

A selective decrease in REM latency was found at 7 and 21 d 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. 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



**Figure 7.** EEG power spectra (absolute power density) in slow-wave sleep (SWS)-1 (a–c) and SWS-2 phase (d–f) at 7 d (a, d), 21 d (b, e), and 180 d (c, f) after MDMA treatment. Each point represents mean values  $\pm$  S.E.M. ( $n=8$ ) of 1 Hz bins in a 2-h recording period beginning at light onset. \* Significantly different compared to the saline group ( $p<0.05$ ). —■—, Sal; -▲-, MDMA.

5-HTT expression together with decreased 5-HT availability observed 21 d 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 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 tuberomammillary nucleus, parallel with reduced REM latency at 7 and 21 d 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 decrease in REM latency induced by MDMA may be explained by a decreased inhibition of SCN by 5-HT, and consequently an earlier activation of SCN. 5-HT 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 5-HT<sub>7</sub> and

5-HT<sub>1B</sub> 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 in 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 fibre density in the SCN could be one possible explanation for decreased REM latency.

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 criteria (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). MDMA has been shown to disrupt the circadian cycle *in vivo* and 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 finding of altered 5-HTT densities in the SCN after MDMA treatment further supports the role of SCN in sleep disturbances caused by MDMA.

Despite the reduced REM latency, we could not find significant changes in the amount of REM sleep after REM onset. The firing rate of DRN neurons is almost absent and is also decreased in the MRN 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 d. This finding is consistent with the increase in delta power caused by 5-HT<sub>2A</sub> receptor antagonists (Dugovic *et al.*, 1989; Monti and Monti, 1999; Sebban *et al.*, 2002; Ursin, 2002). Interestingly, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub> 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<sub>2A</sub> receptors has been suggested on the basis of pharmacological studies (Monti and Monti, 1999; Sebban *et al.*, 2002; Ursin, 2002)

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

In one study, sleep alterations were compared in MDMA users and control subjects. 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 from the last ecstasy use was very heterogeneous in the sample (Allen *et al.*, 1993).

In conclusion, transient changes in the terminal and cellular regions of the ascending serotonergic system, and in parallel some altered sleep functions, were found after a single dose of MDMA. These data provide further evidence for the connection between altered serotonergic functions and sleep disturbance. Despite prolonged serotonergic damage in the hippocampus, all functional changes in sleep were normalized together with recovery of fibre density in most brain areas 180 d after MDMA treatment.

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### Statement of Interest

None.

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