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Research report

MDMA and fenfluramine alter the response of the circadian clock to a serotonin agonist in vitro

Stephany M. Biello*, Richard I. Dafters

Department of Psychology, 58 Hillhead Street, Glasgow University, Glasgow G12 8QB, Scotland, UK

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Abstract

The substituted amphetamine drugs, 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') and fenfluramine, are known to damage 5-HT neurons in the brain of animals. However, little is known about the drugs' effects on circadian rhythmicity which is known to be influenced by serotonergic input to the suprachiasmatic nuclei. In the present study, we tested the ability of MDMA and fenfluramine treatment to alter the ability of the circadian clock to reset in response to an agonist of the 5-HT1A and 5-HT7 receptor subtypes soon after treatment with the drugs, and then again at 20 weeks. Coronal hypothalamic slices containing the suprachiasmatic nuclei (SCN) were prepared from rats and 3-min recordings of the firing rate of individual cells were performed throughout a 12-h period. The ability of the 5-HT agonist, 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT), to cause a phase advance in the firing pattern of SCN neurons was assessed in slices from control animals and those pretreated with MDMA or fenfluramine (10, 15 and 20 mg/kg administered on successive days) 6–10 days or 20 weeks previously. Phase advances to 8-OH-DPAT in the slice were attenuated by pretreatment with MDMA or fenfluramine at both drug-test intervals. Our study demonstrates that repeated exposure to MDMA or fenfluramine may interfere with the ability of serotonin to phase shift the circadian clock in the rat. It is possible that such an effect may be responsible for some of the clinical changes, such as sleep disorders and mood changes, sometimes reported by human users of the substituted amphetamines.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Circadian; Ecstasy; 3,4-Methylenedioxymethamphetamine; Serotonin; Brain slice; Suprachiasmatic nucleus

1. Introduction

The substituted amphetamines, 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') and fenfluramine, are selective serotonin (5-HT) neurotoxins in animals as evidenced by loss of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) from widespread sites throughout the brain [31,36,37] and by neurodegeneration studies using immunocytochemical and reactive gliosis techniques [14]. Such findings have fueled fears that MDMA, which is a widely used recreational drug, and fenfluramine which has been extensively prescribed as an anti-obesity drug, may cause similar damage in humans [35]. Currently, there is little direct evidence of human neurotoxicity although recent studies have reported that prior MDMA use correlates with reduced binding of a selective 5-HT transporter PET ligand [20], with electroencephalographic (EEG) changes in the brain [11], and with reductions in cerebrospinal fluid (CSF) levels of 5-HIAA [19].

5-HT is a widespread neurotransmitter known to affect mood, anxiety, aggressiveness, cognitive function and many regulatory metabolic functions. At least some of these effects are mediated directly or indirectly through serotonergic input to the hypothathalamic–pituitary–adrenocortical axis (HPA). Given this fact, and the evidence for neurotoxicity, there have been surprisingly few reports

Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; PRC, phase response curve; SCN, suprachiasmatic nuclei; ZT, Zeitgeber time

*Corresponding author. Tel.: +44-141-330-3625; fax: +44-141-330-5089.

E-mail addresses: stephany@psy.gla.ac.uk and s.biello@psy.gla.ac.uk (S.M. Biello).

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of neuropsychiatric consequences of substituted amphetamine use sufficient to arouse clinical concern. This may partly reflect the fact that the appearance of functional disorders may require the loss of a high proportion of 5-HT neurons and/or that the doses administered in animal neurotoxicity studies are typically much higher than the human recreational dose. Also, subclinical effects of the drugs may only be apparent using fairly sensitive and specific laboratory tests. Where such tests have been used, selective memory dysfunction [22,23,26] and impulsivity changes [22] have been found. With the exception of thermoregulation, which has been extensively studied because of the role of hyperthermia in the deaths and acute health problems associated with recreational MDMA use [10,16,29], relatively little attention has been paid to the effects of the amphetamine analogues on fundamental metabolic regulatory mechanisms. An important omission is the lack of data concerning the drugs' effects on circadian rhythmicity which is known to be strongly influenced by serotonergic input to the suprachiasmatic nucleus. In humans, rhythm disorders are linked with sleep disorders, difficulties with concentrating, and mood changes, as well as decrements in mental and physical performance [12,18,32].

The hypothalamic suprachiasmatic nuclei (SCN) function as an endogenous circadian pacemaker in mammals [33], and generate a self-sustained oscillation in vitro. This oscillation may be measured in the hypothalamic brain slice preparation as a rhythm in firing rate that can be observed for three to four ~24-h cycles [28]. The daily oscillation in the firing rate of SCN neurons provides a unique system in which to examine the effect of drugs and other stimuli on the circadian clock.

Serotonergic cells in the raphe project to the suprachiasmatic nucleus, and serotonin modulates the activity of the circadian clock [24]. In the rat, serotonin agonists such as quipazine, 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) and 5-CT can reset the phase of the circadian clock when administered directly to the SCN in this hypothalamic preparation [27]. The pattern of resetting is similar in timing and direction to what are termed non-photic stimuli [25]. These phase shifts can be blocked by the 5-HT7 antagonist ritanserin, but not pindolol (which is thought to act on the 5-HT1A subtype). Therefore it appears that the receptor mediating this direct effect on SCN neurons is probably the 5-HT7 subtype that is positively coupled to adenylate cyclase [30]. Depletion of serotonin within the brains of rats produces alterations in circadian parameters. Parachlorophenlalanine altered the free running rhythm in constant light, and lesions of the raphe show disruption of the daily pattern of activity [24].

Since serotonin is involved in the function of the circadian clock, and MDMA administration has been shown to damage serotonin fibers in the raphe, we tested the ability of MDMA and fenfluramine treatment to alter the ability of the circadian clock to reset in response to an agonist of the 5-HT1A and 7 receptor subtypes soon after treatment, and then again at 20 weeks.

2. Materials and methods

2.1. Animals and injections

Male Wistar rats (Charles River, UK) were housed under a light:dark schedule of 12:12 h and given food and water ad libitum. Some rats ($n=15$) received no MDMA. Others were injected subcutaneously with MDMA ($n=14$) (gifted from the National Institute of Health, NIH, USA) or fenfluramine ($n=15$) (Sigma-Aldrich, Fancy Road, Poole, Dorset BH12 4QH, UK) during the light portion (between Zeitgeber time 9–11; Zeitgeber time (ZT) was defined as ZT 12 being the projected time of lights off in the animal room) over 3 consecutive days. The dose of the MDMA or fenfluramine (Fen) was increased over each of the 3 days such that each rat received 10 mg/kg on day 1, 15 mg/kg on day 2, and 20 mg/kg on the final day of injection. Rats were sacrificed for recording after a short interval (6–10 days after MDMA or Fen treatment) or a longer interval (150–155 days). The 'Principles of Laboratory Animal Care' (NIH publication no. 85-23) were followed.

2.2. Tissue preparation

Rats were administered an overdose of halothane anaesthesia and decapitated during the phases when this manipulation does not induce phase shifts, between ZT 2 and 5. Hypothalamic slices (500 μm) containing the SCN were placed in a gas-fluid interface slice chamber (Medical Systems BSC with Haas top), continuously bathed (1 ml/min) in artificial cerebrospinal fluid (ACSF) containing 125.2 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM glucose. ACSF (pH 7.4) was supplemented with an antibiotic (gentamicin, 0.05 g/l) and a fungicide (amphotericin, 2 mg/l) and maintained at 34.5°C. Warm, humidified 95% oxygen:5% carbon dioxide was continuously provided.

2.3. Electrophysiological recordings

Extracellular single unit activity of SCN cells was detected with glass micropipette electrodes filled with 2 M NaCl, advanced through the slice using a hydraulic microdrive. The signal was fed into an amplifier for further amplification and filtering, and was continuously monitored by an oscilloscope and audio monitor. Firing rate and interspike interval data were analyzed using data acquisition software and a customized program for calculation of

descriptive statistics. The person recording cells was blind to all treatments.

2.4. Drugs and treatments

All treatments to the hypothalamic slice were applied as a 200-nl microdrop to the SCN area at least 1 h after slice preparation, on the same day as slice preparation. Recordings were performed for at least 12 h, during ZT 0–12 of the second 24 h in vitro (i.e. ZT 24–36). Treatments were warmed to 34.5°C.

The concentrations of 8-OH-DPAT (10 μM in ACSF), of neuropeptide Y (NPY; 175 μM in ACSF), of pituitary adenylate cyclase-activating peptide (PACAP-38; 1 μM in ACSF) are similar to the dose used in previous in vitro studies [8,15,27]. All drugs were purchased from Sigma (Poole, Dorset, UK) and applied in a 200-nl volume. They were manually placed onto the area of the SCN in the brain slice using a Hamilton 1-μl syringe (microdrop application). 8-OH-DPAT was applied at ZT 8; NPY and PACAP were applied at ZT 6.

Thus there were ten conditions of treatment:

Control 1: Slices prepared from rats receiving no MDMA or fenfluramine, and then either remaining untreated on the 1st day in vitro ($n=6$) or treated with ACSF applied at ZT 8 on the 1st day in vitro ($n=3$).
8-OH-DPAT: Slices prepared from rats receiving no MDMA or fenfluramine prior to recording and treated with 8-OH-DPAT at ZT 8 on the 1st day in vitro ($n=6$).
Control 2: Slices prepared from rats treated with MDMA ($n=3$) or fenfluramine ($n=3$), then recorded ~1 week later.
Control 3: Slices prepared from rats treated with MDMA ($n=3$) or fenfluramine ($n=3$), then recorded ~20 weeks later.
MDMA and DPAT at 1 week: Slices prepared from rats treated with MDMA, then ~1 week later receiving 8-OH-DPAT at ZT 8 on the 1st day in vitro ($n=5$).
MDMA and DPAT at 20 weeks: Slices prepared from rats treated with MDMA, then ~20 weeks later receiving 8-OH-DPAT at ZT 8 on the 1st day in vitro ($n=5$).
Fen and DPAT at 1 week: Slices prepared from rats treated with fenfluramine, then ~1 week later receiving 8-OH-DPAT at ZT 8 on the 1st day in vitro ($n=6$).
Fen and DPAT at 20 weeks: Slices prepared from rats treated with fenfluramine, then ~20 weeks later receiving 8-OH-DPAT the 1st day in vitro ($n=5$).
NPY at 1 week: Slices prepared from rats treated with MDMA, then ~1 week later receiving NPY at ZT 6 on the 1st day in vitro ($n=4$).
PACAP at 1 week: Slices prepared from rats treated with MDMA, then ~1 week later receiving PACAP at ZT 6 on the 1st day in vitro ($n=4$).

2.5. Data analysis

Data were initially grouped into 1-h bins and an analysis of variance test was used to determine if any bins differed from the others. If the analysis of variance test indicated significant differences ($P<0.05$) data were smoothed using 1-h running means with a 15-min lag [8]. Phase shifts of individual slices were measured relative to the average phase of peak firing of the six untreated control slices.

3. Results

3.1. Controls

Untreated control slices showed peak firing rates between ZT 6.4 and ZT 7.3 ($n=6$, mean peak±S.E.M., 6.8±0.2). The phase of the peaks in slices that received applications of ACSF did not significantly differ from those in untreated slices ($n=3$, mean peak±S.E.M., 6.8±0.1). The phase of the peaks seen in slices prepared from animals pretreated with MDMA or fenfluramine and recorded 1 week (controls 2) or 20 weeks (controls 3) after the treatment did not significantly differ from those seen in untreated slices (controls 2: MDMA pretreated recorded at 1 week, $n=3$, mean±S.E.M., 6.8±0.1; fenfluramine pretreated, recorded at 1 week, $n=3$, mean peak±S.E.M., 6.9±0.1; controls 3: MDMA pretreated, recorded at 20 weeks, $n=3$, mean peak±S.E.M., 6.8±0.1; fenfluramine pretreated, recorded at 20 weeks, $n=3$, mean peak±S.E.M., 6.9±0.1).

3.2. 8-OH-DPAT

There was a significant difference in the mean peak time of the slices depending on treatment ($P<0.001$, ANOVA).

Application of 8-OH-DPAT at ZT 8 produced slices with peaks ranging from 3.2 to 4.0. This was significantly different than peaks seen in control slices ($P<0.05$, Bonferroni; $n=6$, mean peak±S.E.M., 3.6±0.1) (Fig. 1). Therefore 8-OH-DPAT produced a phase advance shift in the slice of 3.3±0.1 h when compared with untreated controls.

3.3. MDMA and DPAT at 1 week

There was a significant difference in the mean peak time between slices treated with 8-OH-DPAT prepared from animals pretreated with MDMA, and recorded ~1 week later. This was different from the peak observed in control group 1 and the group given 8-OH-DPAT ($P<0.05$, Bonferroni; $n=5$, mean peak±S.E.M., 5.1±0.2). Therefore the phase advance shift seen in response to 8-OH-DPAT

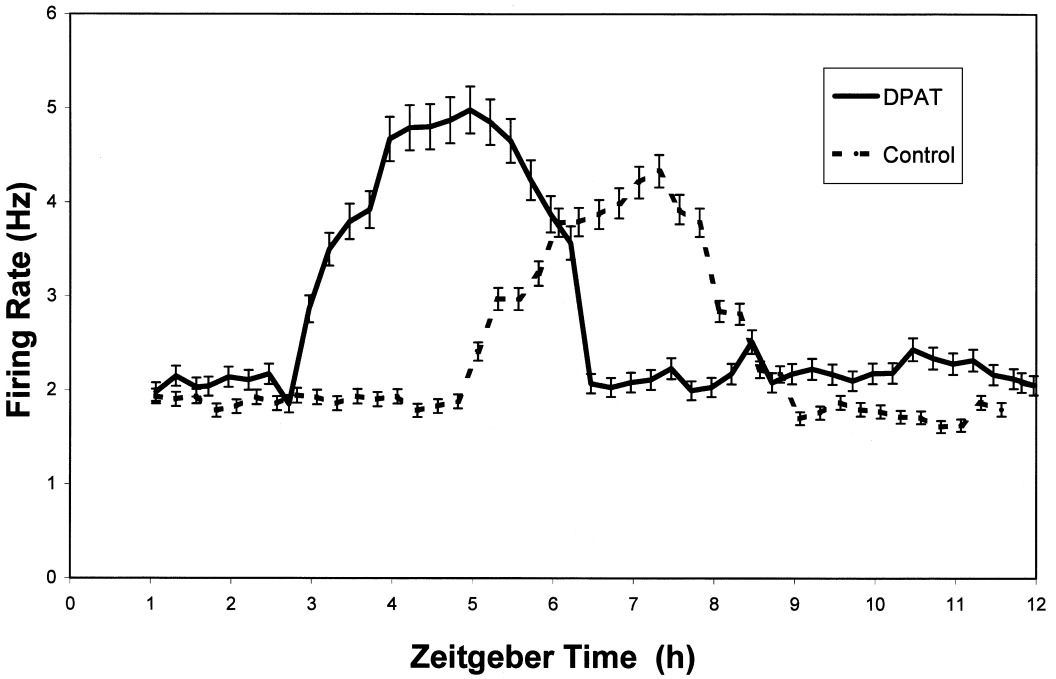

Fig. 1. Peak times in slices treated with DPAT (200-nl microdrop, 10 μM) are advanced compared with untreated control slices. The lines indicate results from the running mean smoother for all cells recorded from untreated control slices (slice number=6) or slices treated with 8-OH-DPAT at ZT 8 (slice number=6). ZT 12 is defined as the time of lights off in the animal's previous light:dark cycle. This graph shows data from ZT 0–12 recorded during the 2nd day in vitro.

after animals had been pretreated with MDMA, and the recording made ~1 week later, was 1.3±0.2 h when compared with untreated controls (Fig. 2).

3.4. MDMA and DPAT at 20 weeks

Slices prepared from animals left for 20 weeks after

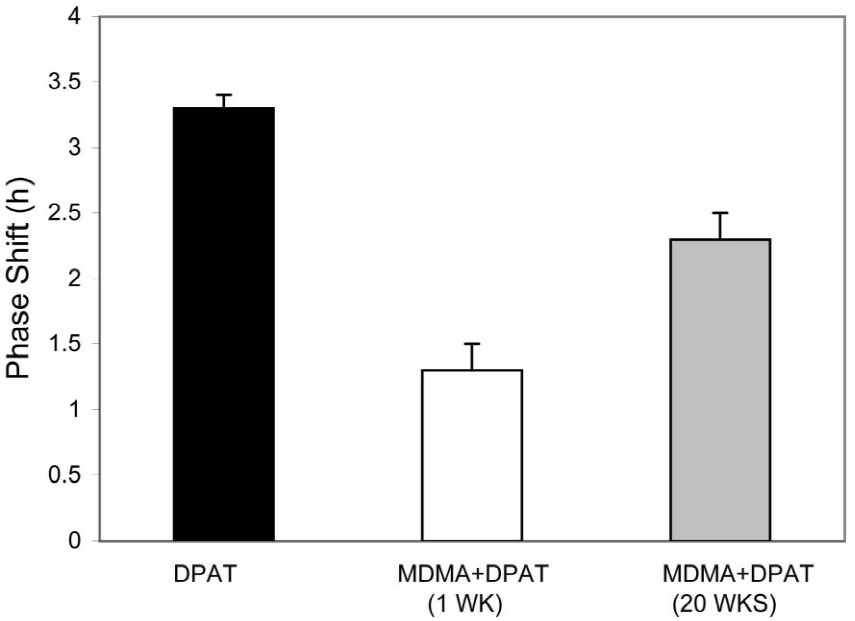

Fig. 2. Phase shifts to DPAT are attenuated in slices prepared from animals pre-treated with MDMA. Histogram showing phase shifts when treated with DPAT (n=6) or MDMA and DPAT at 1 week (n=5), or MDMA and DPAT at 20 weeks (n=5). Significance at $P<0.05$, mean phase shift±S.E.M.

MDMA, and subsequently treated with 8-OH-DPAT, showed mean peaks which were significantly different from that observed in the groups given 8-OH-DPAT or MDMA and DPAT at 1 week ($P<0.05$, Bonferroni; $n=5$, mean peak±S.E.M., 4.5±0.2). Therefore the phase advance shift seen in response to 8-OH-DPAT after animals had been pretreated with MDMA, and the recording made ~20 weeks, later was 2.3±0.2 h when compared with untreated controls (Fig. 2).

3.5. Fen and DPAT at 1 week

There was a significant difference in the mean peak time between slices treated with 8-OH-DPAT prepared from animals pretreated with fenfluramine, and recorded ~1 week later. These were different from the peak observed in the control 1 group and the group given 8-OH-DPAT ($P<0.05$, Bonferroni; $n=6$, mean peak±S.E.M., 5.6±0.3). Therefore the phase advance shift seen in response to 8-OH-DPAT after animals had been pretreated with fenfluramine, and recorded ~1 week later, was 1.2±0.3 h when compared with untreated controls (Fig. 3).

3.6. Fen and DPAT at 20 weeks

Slices prepared from animals left for 20 weeks after fenfluramine, and subsequently treated with 8-OH-DPAT, showed peaks which were significantly different from that observed in the groups given 8-OH-DPAT or Fen and 8-OH-DPAT at 1 week ($P<0.05$, Bonferroni; $n=5$, mean peak±S.E.M., 4.4±0.2). Therefore the phase advance shift seen in response to 8-OH-DPAT after animals had been pretreated with fenfluramine, and the recording made ~20 weeks later, was 2.5±0.2 h when compared with untreated controls (Fig. 3).

3.7. MDMA and NPY or PACAP at 1 week

There was a significant difference in the mean peak time between slices treated with NPY prepared from animals pretreated with MDMA, and recorded ~1 week later. These were different from the peak observed in the control 1 group (NPY, $P<0.05$, Bonferroni; $n=4$, mean peak±S.E.M., 3.6±0.1; PACAP, $P<0.05$, Bonferroni; $n=4$, mean peak±S.E.M., 3.1±0.1). Therefore the phase advance shift seen after animals had been pretreated with MDMA, and recorded ~1 week later was 3.2±0.1 h in response to NPY and 3.7±0.1 h in response to PACAP when compared with untreated controls.

4. Discussion

Administration of MDMA and fenfluramine produces selective degeneration of serotonergic nerve terminals and a significant depletion of serotonin in both rats and non-human primates [6,31,36]. In addition, a serotonergic contribution to the regulation of the circadian clock has been well documented [24]. We have here investigated the effect of repeated MDMA or fenfluramine treatment on the response of the circadian clock to serotonin.

Rats pretreated with MDMA or fenfluramine phase shifted significantly less to the 5-HT1A/7 serotonin receptor agonist 8-OH-DPAT than untreated animals. While

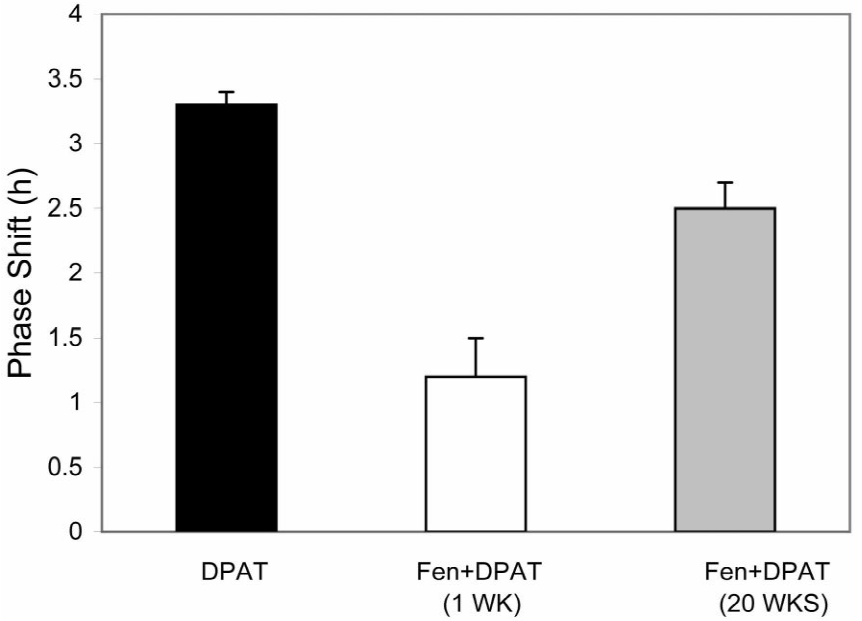

Fig. 3. Phase shifts to DPAT are attenuated in slices prepared from animals pre-treated with fenfluramine. Histogram showing phase shifts when treated with fenfluramine ($n=6$) or Fen and DPAT at 1 week ($n=6$) and Fen and DPAT at 20 weeks ($n=5$). Significance at $P<0.05$, mean phase shift±S.E.M.

the decrement was not as great after an interval of ~20 weeks, it was still significantly different from the response of untreated controls. The mechanism by which this occurs is unclear. Alteration of behaviour in response to serotonin receptor agonists after treatment with the drugs could occur for a number of reasons. There could be alteration in the number or sensitivity of receptors mediating the response. It is also possible that MDMA could alter the activity of another transmitter necessary for the phase shifting action of the agonist.

The phase shifting effect of 8-OH-DPAT may be mediated by the 5-HT1A/7 receptor in the SCN, although some effects of serotonin on clock function are thought to be mediated by the 5-HT1B receptor [30]. It is possible that pretreatment with substituted amphetamines could alter the response of the circadian clock to the serotonin agonist by altering the number and distribution of serotonin receptors in the clock area. Treatment with MDMA has been shown to increase 5-HT1A (but not 5-HT7) receptor number in the hypothalamus [3]. While this is indicative of the hypothalamus as a whole, there exist no data specifically looking at receptor changes in the SCN after MDMA treatment. Other brain areas including the hippocampus and brainstem exhibit decreased 5-HT1A receptor density after repeated treatment with MDMA [2].

Changes in the sensitivity of 5-HT receptors in response to serotonergic denervation of hypothalamic neurons have been reported. While increased sensitivity of receptors has been reported after destruction of serotonin neurons via 5,7-DHT [39], others have reported more variable effects after administration of MDMA. For example, repeated administration of MDMA did not alter the behavioural responses of rats to a 5-HT2A/C receptor agonist [13]. Further it has been shown that depletion of serotonin differentially effects behaviours controlled by various receptor subtypes [7]. Finally, serotonin receptor down regulation in response to MDMA has also been reported [34]. MDMA and Fen may act as indirect 5-HT agonists which primarily release 5-HT (and may block uptake to some extent). The excess tonus in the synaptic cleft could downregulate the 5-HT receptors involved in the shifts to DPAT. Thus we may see a smaller response to DPAT after MDMA pretreatment because we have downregulated the 5-HT receptor.

It is also possible that destruction of serotonin neurons in the raphe that project to the SCN may interfere with other colocalised transmitters. For example, a small number of cells which contain both gamma-aminobutyric acid (GABA) and serotonin have been reported in the raphe nucleus of the rat [38]. It is likely that GABA is an important neurotransmitter in the circadian system. The SCN receives extensive GABAergic innervation and nearly all of the neurones in the SCN are GABA-producing [21]. In addition, the SCN shows changes in GABA content and glutamic acid decarboxylase activity under a light/dark cycle, and changes in the GABA content in animals kept under constant darkness [1]. These suggest that extracellular concentration of GABA varies within the SCN region over time. It is possible that the loss of the input of other transmitters co-localised with serotonin, such as GABA, could alter coupling between receptor and second messenger systems within the SCN, although it is likely this interaction would be complex.

The functional role of serotonin in the circadian system of rats is not fully understood [17]. Numerous studies have shown that serotonin agonists applied to the isolated SCN in vitro during the day produce the phase advance characteristic of non-photic stimuli [28]. Quipazine applied in vitro does not induce c-fos mRNA when applied during the night, as would be expected of a photic stimulus [30]. However, recent data from in vivo studies in the rat suggests a role for serotonin in phase shifts to photic stimuli. Quipazine applied systemically to rats early in the night produced phase delays while application late in the night produced phase advances. Furthermore, quipazine or 5-CT microinjected into the third ventricle produced photic-like shifts and induced expression of c-Fos in the ventral SCN [17]. There are a number of reasons why this difference between in vivo and in vitro might occur. First it is possible that disinhibition by deafferentation within the slice preparation might cause alterations in the actions of serotonin on the clock. In addition it is possible that the systemic injections and even ventricular injections do not affect the SCN directly, but influence other brain areas that project to the clock.

The isolated SCN reset in response to NPY and PACAP when following pretreatment with MDMA. In our study NPY phase advanced the in vitro SCN by ~3.2 h. This advance is similar to previously published shifts to the same dose of NPY (3.6 h) in MDMA naïve animals [8]. The shift seen in response to application of PACAP in animals pretreated with MDMA is also similar to the previously published phase advances seen in MDMA naïve animals of 3.5 h [15]. This suggests that repeated administration of MDMA on the clock does not alter the ability of the pacemaker to reset in response to stimuli by some alteration of the circadian clock in a non-specific way. Further experiments that test the actions of other neurochemicals on the clock after pretreatment with MDMA both in vitro and in vivo may be instructive.

We have demonstrated an alteration in the response of the circadian clock to a serotonin agonist after pretreatment with MDMA and fenfluramine. The phase shifting response had still not recovered at 20 weeks. It is possible that recovery of the response would be seen in animals left longer, as rodents have exhibited regeneration of the serotonin system after MDMA treatment [5]. Even though reinnervation of the SCN after 5,7-DHT lesions in rodents has been shown, it is not complete at 1 year, and evidence fails to show functional recovery [9].

Since alteration in serotonin function has been shown to have effects on entrainment, it is possible that repeated

MDMA administration could interfere with the ability of the clock to synchronize to the environmental cycle of light and dark. It is possible that repeated doses of MDMA and fenfluramine may influence clock function in humans as has been demonstrated here in rodents and that this may be responsible for some of the changes in mood and sleep patterns reported to follow MDMA use in humans [4].

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