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Functional consequences of perinatal exposure to 3,4-methylenedioxymethamphetamine in rat brain

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1 In this study we have examined methylenedioxymethamphetamine (MDMA)-induced toxicity in perinatal rat brain, related this to normal development of serotonin transporter sites (SERT), and determined whether early exposure to MDMA subsequently alters cerebral function in adults.

2 Perinatal development of SERT was visualized and quantified using [^{3}H]-paroxetine binding autoradiography in embryonic and neonatal rat brain from embryonic day 15 (E15) to postnatal day 30 (P30). Cerebral glucose utilization ($lCMR_{glu}$) was measured by 2-deoxyglucose autoradiography in adult rats.

3 [^{3}H]-Paroxetine binding was observed in forebrain from E18. From birth (P0), binding was organized into neocortical columns (75% higher at P10 than in adult) which declined toward adult levels between P20 and P25.

4 MDMA treatment (20 mg kg^{-1} s.c. twice daily for four days) commencing at developmental stages from E15 (treatment given to dams) to P20, had no effect upon [^{3}H]-paroxetine binding measured at P40. Treatments started on P25 or later resulted in significant decreases in [^{3}H]-paroxetine binding ($\geqslant$46%). This was coincident with the development of adult patterns of binding in forebrain.

5 Despite the lack of MDMA-induced neurotoxicity, rats treated *in utero* (E15) showed increased $lCMR_{glu}$ in locus coeruleus (+37%), and in areas receiving ascending noradrenergic innervation, such as anterior thalamus (+44%) and septal nucleus (+24%).

6 These studies confirm that the susceptibility of serotonergic terminals to the neurotoxic properties of MDMA is absent in the immediate perinatal period, but also suggests that *in utero* MDMA exposure produces significant long-term effects on cerebral function by a mechanism as yet unknown.

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Abbreviations: 5-HT, 5-hydroxytryptamine; E, embryonic day; $lCMR_{glu}$, local cerebral glucose utilization; MABP, mean arterial blood pressure; MDMA, 3,4-methylenedioxymethamphetamine; P, postnatal day; SERT, serotonin transporter site

Introduction

In neonatal rat a transient, focally dense serotonergic (5-HT) innervation of forebrain appears around birth and, in neocortex, persists throughout the first 3 to 4 weeks of postnatal life (Bennet-Clarke *et al*., 1991; Boylan *et al*., 2000; D'Amato *et al*., 1987). It has been proposed that this apparent hyperinnervation exerts a trophic influence upon developing hippocampus (Yan *et al*., 1997b) and thalamocortical projections in particular (Boylan *et al*., 2000; Osterheldhaas & Hornung, 1996). Any disruption to serotonergic nerve terminals during this time may have consequences for the normal development of cerebral architectonics and function (Lauder *et al*., 2000; Mazer *et al*., 1997; Yan *et al*., 1997a) and exposure to a variety of serotinergic drugs has been shown to alter normal brain development (Lauder *et al*., 2000; Mazer *et al*., 1997; Young-Davis *et al*., 2000), particularly during critical periods.

Like other amphetamine-related drugs, methylenedioxymethamphetamine (MDMA; 'Ecstasy') possesses neurotoxic properties, but in contrast to other amphetamines the neurotoxicity appears to be most selective for serotonergic nerve terminals whilst catehcholaminergic neurones remain largely intact (Battaglia *et al*., 1987). The evidence that MDMA is toxic to central serotonergic terminals has been derived from several different species of experimental animal, but in adult rat brain, the toxic effects of repeated exposure to MDMA are shown by a marked depletion of 5-HT immunoreactive axon terminals and reduction in 5-HT transporter (SERT) densities (Battaglia *et al*., 1987). Even a single exposure can result in some manifestations of neuronal damage (Colado *et al*., 1997). In contrast, current evidence suggests that the effects of MDMA upon the developing brain are much less dramatic. No evidence of neurotoxicity has as yet been identified in the brains of rats exposed to MDMA *in utero* or during the immediate post-natal period (Aguire *et al*., 1998; Broening *et al*., 1994; Colado *et al*.,

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1997; St Omer *et al*., 1991), although there is some evidence of behavioural, and subtle biochemical effects lasting to maturity (Broening *et al*., 2001).

In this investigation, three parallel studies were conducted to further explore the effects of MDMA upon the developing rat brain. Firstly, we mapped the changing distribution of serotonergic nerve terminals in normal perinatal rat brains using [^{3}H]-paroxetine radioligand binding autoradiogaphy. Secondly, we measured [^{3}H]-paroxetine binding in preparations of neocortical membrane in groups of adult rats which as neonates had been injected with MDMA at different time points, or which had been born to dams treated with MDMA during the latter stages of gestation, in order to investigate whether we could detect any long-lasting effects of perinatal MDMA exposure. Finally, using litter mates of those treated rats used in membrane binding studies, we measured local cerebral glucose utilization ($lCMR_{glu}$) to examine the effects of perinatal MDMA upon functional activity in different areas of adult brain.

Methods

All experiments involving the use of animals were performed under the authority of United Kingdom Animals (Scientific Procedures) Act 1986, Project Licence number 60/2280 and were subject to local ethical scrutiny by senior Named Animal Care and Welfare Officers. MDMA was used under the authority of United Kingdom Misuse of Drugs Act 1971, Licence to be in Possession number 0/MM/3388.

Perinatal development of 5-HT uptake sites in normal rat brain

Untreated neonatal male Sprague–Dawley rats from untreated dams ($n=7$) were sacrificed on postnatal days 0, 5, 10, 15, 20, 25, and 30 ($n=5$ at each time point). The brains were dissected intact, and coronal cyrostat sections at the level of the caudate nucleus were thaw-mounted onto gelatin-coated glass slides. Prenatal brains were obtained from unsexed foetuses harvested from untreated pregnant female rats ($n=3$) sacrificed on gestational day 18, and were similarly sectioned. No more than one animal from a given litter was used at any single time point. The untreated dams provided sections of adult brain for comparison purposes.

At each of the chosen time points in perinatal development, the distribution of 5-HT uptake sites was visualized using [^{3}H]-paroxetine autoradiography using the method as described by De Souza & Kuyatt (1987). Briefly, sections were incubated in buffer containing a saturating concentration (250 pM) of [^{3}H]-paroxetine (specific activity 23.1 Ci mmol^{-1}; NEN, DuPont) for 2 h. Non-specific binding was defined in adjacent sections by [^{3}H]-paroxetine binding in the presence of 4 μM citalopram in the incubation medium. The sections were washed in buffer, dipped in deionized water, then dried rapidly under a stream of cold air. The sections, together with a set of precalibrated [^{3}H]-containing standards (Amersham Microscales), were applied to X-ray film (Amersham, Hyperfilm-^{3}H) in a light-tight cassette at -70°C for 6 weeks.

Autoradiograms were analysed using a computer-based image analysis system (MCID/M4). Local tissue [^{3}H]-concentrations were determined with reference to the precalibrated standards, and ligand binding concentrations calculated. Specific binding of [^{3}H]-paroxetine in neocortex was determined by the subtraction of non-specific binding from corresponding total binding concentrations in adjacent sections. Data were analysed using Student's *t*-test for grouped data with acceptable levels of significance set at $P<0.05$.

Prenatal MDMA treatment

Time-mated female Sprague-Dawley rats were injected subcutaneously (s.c.) with either MDMA (20 mg kg^{-1}; $n=5$) or an equal volume of saline (0.2 ml; $n=5$) twice daily on four consecutive days commencing on gestational day 15 (E15). Throughout the pregnancies, both before and after treatment, the female rats were checked twice daily by trained animal technicians to ensure that no individual had visible signs of obstetric complications. Dams were allowed to give birth (around E22) and suckle their pups normally. Offspring were weaned on postantal day 22 (P22), separated according to sex, ear-marked to allow identification of the source litter, and housed under normal conditions with no further intervention until they reached 40 days of age when they were used for neocortical membrane receptor binding assays (described below), or around 90 days when they were used for the measurement of cerebral glucose utilization (described below) and [^{3}H]-paroxetine binding autoradiography (described above). Following the weaning of their offspring, the dams were sacrificed, the brains dissected intact, and processed for [^{3}H]-paroxetine membrane binding.

Postnatal MDMA treatment

Neonatal male rats (from a total of 10 untreated dams) were injected (s.c.) with either MDMA (20 mg kg^{-1}) or saline (0.2 ml) twice daily on four consecutive days, commencing on postnatal days 10, 15, 20, 25, or 30 ($n=5$ or 6 at each time point, with no more than one from each litter). All animals were sacrificed on postnatal day 40. Brain tissue was dissected from frontal cortex, weighed, frozen on dry ice, and stored at -70°C prior to processing for *in vitro* analysis of [^{3}H]-paroxetine-labelled 5-HT uptake sites (Battaglia *et al*., 1987). A further group of offspring were injected with MDMA ($n=5$) or saline ($n=5$) at P90 using the same treatment protocol, sacrificed after 10 days and the brains processed as for [^{3}H]-paroxetine membrane binding in the same way as the neonates.

In a study designed to eliminate the possibility of regrowth of serotonergic terminals following perinatal MDMA treatment, further neonatal rats were injected (s.c.) with either MDMA (20 mg kg^{-1}, $n=10$) or saline (0.2 ml, $n=10$) twice daily on four consecutive days commecning on postnatal day 10. Half of each treatment group was sacrificed on postnatal day 25. The brains of these animals were dissected intact, frozen, and sectioned in a cryostat. Coronal sections at the level of the caudata nucleus were thaw-mounted onto gelain covered glass slides for subsequent autoradiographic [^{3}H]-paroxetine ligand binding analysis of serotonergic uptake sites as described above. The remaining animals were taken around P90 for the measurement of cerebral glucose utilization.

In vitro *membrane receptor binding assays*

Cell membranes were prepared from homogenates of frontal cortex taken from the brains of adult male rats which had been treated either pre- or postnatally. Saturation analysis of [^{3}H]-paroxetine (specific activity 23.1 Ci mmol^{-1}) was performed as described by us previously (Sharkey *et al*., 1991). Briefly, membranes were incubated in buffer (50 mM TRIS-HCL, 120 mM NaCl and 5 mM KCl, pH 7.7) containing six concentrations of [^{3}H]-paroxetine (4–1000 pM) for 2 h at 22°C. Non-specific binding was defined by the addition of 4 μM citalopram to the incubation medium. Samples were rapidly filtered (Brandel) through polyethyleneimine (0.05% in buffer) pre-soaked Whatman GF/C filters, washed with 3×5 ml aliquots of buffer, than analysed by liquid scintillation spectrometry. Data were fitted by least-squares to the non-linear equation

$$B = B_{max}.F^n/K_D + F^n \quad (1)$$

where B = the amount of [^{3}H]-paroxetine bound at the free ligand concentration, F, and n is the Hill slope, using the curve fitting program, 'GraphPad Prism'. Statistical analysis was performed using ANOVA with *post hoc* Scheffé test to allow multiple pair-wise comparisons. Statistical significance was set at $P<0.05$.

Measurement of lCMRglu

Mature male offspring ($\geqslant$90 days) from dams treated during gestation with MDMA ($n=5$) or dams treated with saline ($n=5$), and rats which themselves had been treated with MDMA ($n=5$) or saline ($n=5$) at 10 days of age, were subsequently prepared for the measurement of local cerebral glucose metabolism (lCMR$_{glu}$). On the day of the experiment, the rats were anaesthetized with halothane (1.5% in a gas mixture of 70% nitrous oxide, 30% oxygen) to allow the insertion of polythene cannulae into both femoral arteries and veins. The incision sites were infiltrated with local anaesthetic and the wound sutured closed. The rats were lightly restrained and allowed to recover for at least 2 h before further experimental procedures.

Measurements of lCMR$_{glu}$ were performed using the fully quantitative [^{14}C]-2-deoxyglucose (Sokoloff *et al*., 1977) autoradiographic technique. In keeping with our previous studies (Kelly *et al*., 1993), only one rat from each appropriately treated dam was included in the experimental groups, in order to avoid 'nesting' of variables from non-independent observations. The lCMR$_{glu}$ measurement protcols were in complete accordance with the methodology as originally published and as previously detailed from this laboratory (Kelly *et al*., 1995). The measurement was initiated with a 30 s intravenous injection of tracer (40 μCi in 0.75 ml saline). Over the subsequent 45 min, a total of 14 timed arterial blood samples were collected at predetermined intervals and centrifuged to separate plasma. Aliquots of each plasma sample were taken for the determination of [^{14}C] concentrations (20 μl) and glucose levels (10 μl) by liquid scintillation analysis and semi-automated glucose oxidase assay (Beckman) respectively. At the end of the measurement period the rats were killed with a rapid intravenous injection of barbiturate (Euthatal), the brains dissected intact, frozen to −40°C in precooled isopentane, and sectioned in a cryostat for the preparation of autoradiograms.

Sections adjacent to those used for autoradiographic measurement of lCMR$_{glu}$ were thaw-mounted onto gelatin covered glass slides and stored at −70°C for subsequent [^{3}H]-paroxetine autoradiographic binding analysis of 5-HT uptake sites. The slide-mounted sections were prepared for autoradiography according to the protocol described above (De Souza & Kuyatt, 1987), with the addition of preliminary wash procedures incorporated in order to remove [^{14}C]-tracers from the tissue (Sharkey *et al*., 1991).

Analysis of [^{14}C]-2-deoxyglucose autoradiograms was performed using a computer-based image analysis system (MCID/M4). Tissue isotope concentrations were measured from autoradiographic images of brain tissue, relative to appropriate [^{14}C]-containing standards (Amersham Microscales), and lCMR$_{glu}$ calculated using the operational equations for the techniques (Sokoloff *et al*., 1977). Data were analysed using Student's *t*-test for grouped data with acceptable levels of significance set at $P<0.05$.

Results

Ontogeny of 5-HT uptake sites

From postnatal day 0 through to 20, high density [^{3}H]-paroxetine binding was evident in neocortex (Figure 1) in a pattern that was both laminar and columnar in organisation. By postnatal day 25, these areas of high density had largely disappeared, and as in the adult brain, cortical [^{3}H]-paroxetine binding was more homogeneously distributed. Although there was some evidence of [^{3}H]-paroxetine binding in prenatal neocortex (gestational day 18), because of the thinness of the cortical mantle it was not possible to determine how this was organized with respect to identifiable anatomical structures. Quantification of the autoradiographic images revealed that the high density of [^{3}H]-paroxetine binding in neocortex (layer IV) represented levels which were greater than that found in adult brain (65 ± 4 fmol mg^{-1}), reaching a peak at P10 (114 ± 9 fmol mg^{-1}; $P<0.05$). Interestingly, [^{3}H]-paroxetine binding in adjacent areas of

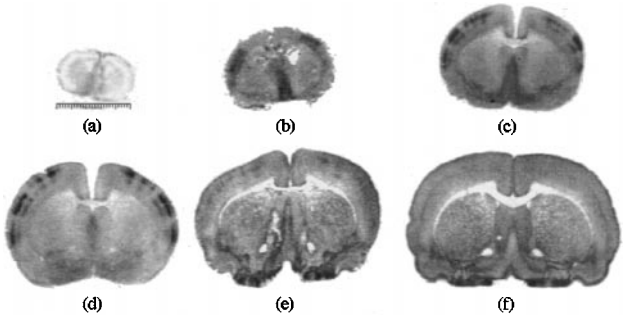

Figure 1 [^{3}H]-paroxetine radioligand binding autoradiography in coronal sections of the rat brain at the level of the caudate nucleus. Sections were taken from animals at (a) E18, (b) P0, (c) P5, (d) P10, (e) P25, and (f) adult (dams of E18 foetuses) and show total binding from which non-specific binding was subtracted for quantification. Scale bar on Figure 1a indicates 5 mm. The same scale was maintained for all other images.

cortex, between the patches, was not significantly different from adult levels.

Effects of MDMA upon [^{3}H]-paroxetine binding

Analysis of [^{3}H]-paroxetine binding isotherms for membrane preparations revealed the presence of an apparently homogeneous population of binding sites in both saline-treated control animals and in animals which had been exposed to MDMA prenatally or at different time points during their postnatal development (Table 1). Hill coefficients were not significantly different from unity, ranging from 0.97 to 1.00. No differences in the density (B_{max}) of [^{3}H]-paroxetine binding sites were found in brains of rats which had been treated with MDMA for periods before postnatal day 25 (Table 1; Figure 2) when compared to saline-treated controls. However, significant reductions were found in the two groups of rats in which MDMA treatment had commenced on postnatal days 25 (−46%) and 30 (−63%) (Table 1; Figure 2). The decreases were not however as marked as those found in rats treated with MDMA at P90, where the B_{max} decreased by 90% (Table 1). In dams treated with MDMA whilst pregnant, membrane binding analysis revealed that [^{3}H]-paroxetine binding was reduced by about 89% ($B_{max} = 3.6 \pm 2.0$ fmol mg tissue^{-1}) when compared to saline-treated females ($B_{max} = 34.4 \pm 0.7$ fmol mg tissue^{-1}).

Analysis of [^{3}H]-paroxetine autoradiograms from animals treated with MDMA on postnatal day 10, and sacrificed on postnatal day 25, showed neither qualitative nor quantitative differences in [^{3}H]-paroxetine-labelled binding sites between treated and control rats. In both groups of rats the adult pattern of neocortical binding densities was evident (as illustrated in Figure 1). In particular, there was no evidence for any delay in the normal disappearance of high density neocortical [^{3}H]-paroxetine-labelled patches in MDMA-treated rats which might have been indicative of an initial loss of 5-HT terminals.

[^{3}H]-Paroxetine binding autoradiography in forebrain sections adjacent to those used for the measurement of $lCMR_{glu}$ in the mature offspring (P90) of dams treated with MDMA starting on gestational day15 (E15), revealed neither qualitative nor quantitative changes in SERT when compared to offspring from saline-treated dams. Neither was there any difference in [^{3}H]-paroxetine binding autoradiography between rats treated with either saline or MDMA at P10. These observations made at P90 are consistent with the data determined by membrane binding when similarly treated animals were sacrificed at P40.

Local cerebral glucose utilization

In general, there was a global tendency for $lCMR_{glu}$ in the mature offspring of MDMA-treated dams to be higher (but also more variable) than that found in the offspring of saline-treated dams, but significant increases were found only in nine out of the 25 anatomically discrete and functionally diverse areas of the brain included for analysis in this study (Table 2). In the hindbrain, $lCMR_{glu}$ was increased by around 20% in a number of nuclei (inferior olive; nucleus ambiguus; trigeminal nucleus), but the largest differences were found in the locus coeruleus (+37% from control). In hippocampus, where we have previously reported metabolic dysfunction following exposure to MDMA in the adult rat (Sharkey *et al*., 1991), we found significant increases in $lCMR_{glu}$ only in the subiculum (+29%). In the remainder of the forebrain, alterations in $lCMR_{glu}$ were limited to the anterior thalamus (+44%), hypothalamus (+25%), septal nucleus (+25%) and globus pallidus (+26%) (Table 2). There were no significant changes in $lCMR_{glu}$ in rats treated at 10 days of age (Table 2).

There was no evidence from the physiological variables measured at the time of the $lCMR_{glu}$ experiments that rats exposed to MDMA *in utero* responded differently to the stress of the surgical procedures used in setting up the animals for experimentation. In particular, there was no evidence of hyperventilation or hypertension, and circulating glucose concentrations and glucocorticoid levels were not significantly different from control (Table 3).

Discussion

Previous qualitative studies have suggested that the development of cortical serotonergic terminals in rat is characterized by the transient appearance of dense patches of innervation which are highly focal and arranged in both a laminar and columnar pattern. Using an autoradiographic approach similar to that used here, D'Amato *et al*. (1987) described dense patches of binding in cortex which first appeared at P3, peaked during the second week of postnatal life, and had disappeared after P21. Whilst our present study is largely in

Table 1 [^{3}H]-paroxetine binding in membrane preparations from neocortex of rats treated at different stages in development with either saline or MDMA

Age at start of treatment	*Saline control*			*MDMA-treated*		
	K_D (pM)	B_{max} (fmol mg^{-1})	n	K_D (pM)	B_{max} (fmol mg^{-1})	n
E15	39.1±1.8	36.6±0.9	5	37.2±1.3	34.6±1.0§	5
P10	38.0±2.0	38.0±1.0	6	40.8±4.7	35.2±1.7§	6
P15	40.5±0.6	38.3±1.3	5	39.0±0.5	34.4±0.7§	6
P20	39.3±1.6	37.8±1.1	5	40.7±2.1	34.3±1.5§	6
P25	33.4±0.6	34.4±0.8	6	38.1±4.2	18.6±0.8*§	5
P30	38.5±0.9	35.0±0.8	6	37.5±1.3	13.0±0.6*§	5
P90	37.3±3.8	35.5±2.3	5	35.8±8.3	3.5±1.2*	5

Data are presented as mean±s.e.m. All data (with the exception of P90) were determined at P40. Data from rats treated at P90 were determined 10 days after treatment. *, B_{max} sifnificantly different from saline control (treated at the same age); §, B_{max} significantly different from *P*90 ($P<0.05$; ANOVA with *post hoc* Scheffé test for multiple comparisons). There were no significant differences in B_{max} between groups treated with saline.

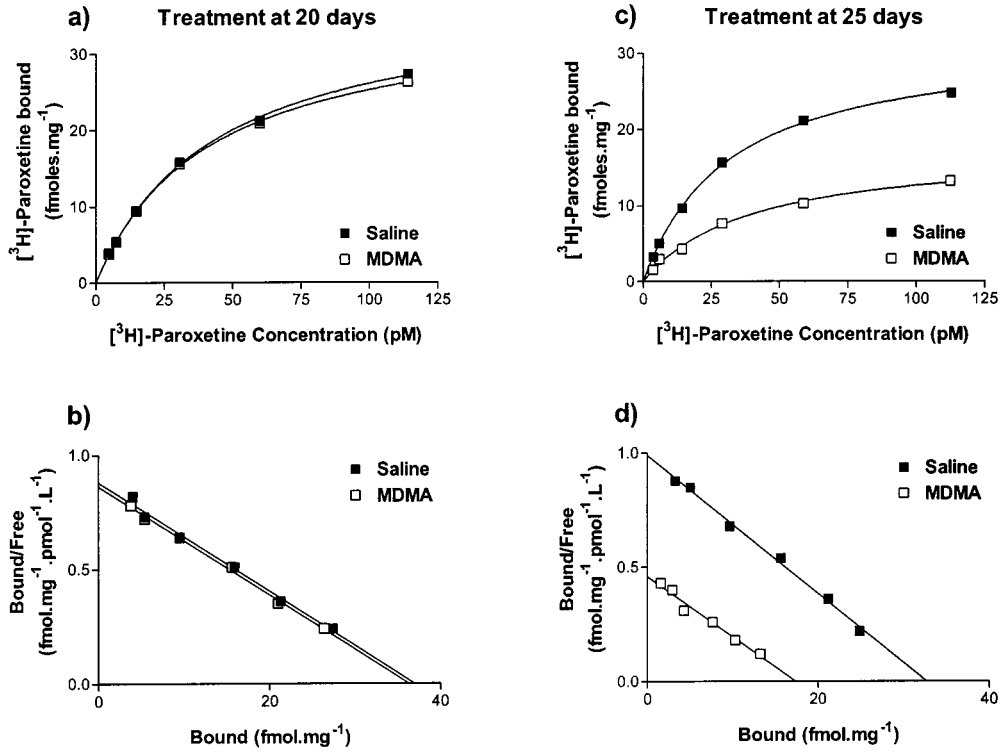

Figure 2 Saturation isotherms (a,c) with Scatchard plots (b,d) of [^{3}H]-paroxetine binding to neocortical cell membranes prepared from 40 day old rats pretreated with saline or MDMA twice daily for four consecutive days commencing on postnatal day 20 (a,b), and postnatal day 25 (c,d). The data illustrated were derived from single representative animals from each treatment group at both time points.

agreement with this description, we have found evidence that neocortical patches of binding to 5-HT uptake sites are present at an earlier age (at birth), although we were unable to detect specific binding to serotonergic terminals before birth as is suggested by immunohistochemistry (Lidov & Molliver, 1982). We also found that patterns of hyperinnervation continue for longer than reported initially, and in keeping with more recent studies (Boylan *et al*., 2000) a columnar pattern of binding could still be discerned at P25, albeit to a greatly reduced extent. Moreover, the fully quantitative autoradiographic approach used by us, allowed us to confirm the subjective visual impression of hyperinnervation during development (75% greater than adult levels of binding) but also showed that in areas surrounding the focal hyperinnervation, binding levels were not significantly different to those found in adult cortical tissue.

The developmental plasticity in serotonergic projections and synaptic elements which occurs in the brains of rats between gestational day 13 and postnatal day 20, takes place *in utero* during the third trimester in human development (Barpeled *et al*., 1991; Broening *et al*., 2001; DelOlmo & Pazos, 2001), and concerns have been expressed that prenatal exposure of the human foetus to MDMA ingested by the mother might permanently alter the distribution of serotonergic nerve terminals in the brain and induce cerebral dysfunction. Certainly there is evidence that the neurotoxic effects of MDMA described in rats and sub-human primates (Battaglia *et al*., 1987; Ricuarte *et al*., 1988a, b) are paralleled in human users of the drug (McCann *et al*., 1994; 1998; Semple *et al*., 1999), although this continues to be controversial (Kish, 2002). However, there is currently no evidence that perinatal exposure to MDMA produces nerutoxic damage in the developing embryo. In rat, neither prenatal MDMA exposure (Colado *et al*., 1997) nor treatments in the second (Colado *et al*., 1997; Broening *et al*., 1994) to fourth weeks (Aguire *et al*., 1998) of postnatal life result in any evidence of neurotoxicity, and it is only with treatments between P35 and P40 that neurotoxicity was found (Broening *et al*., 2001; Aguire *et al*., 1998).

In keeping with these previous studies, the MDMA treatment protocol reported here produced a marked reduction in 5-HT uptake sties, which is indicative of a loss of serotonergic nerve terminals (Battaglia *et al*., 1987), in the brains of female rats treated on days 15–18 of pregnancy. However, no such lasting depletion of 5-HT uptake sites was evident in the forebrain of mature offspring of these dams 4–6 weeks after birth. Similar treatment given directly to neonatal rats between P10 and P24 had no effect upon paroxetine binding when measured at P40. However in rats in which the treatment commenced on P25 and subsequently, paroxetine binding was markedly and significantly reduced, albeit not to the same extent as that found in adult brain.

Although the exact mechanism underlying MDMA-induced neurotoxicity has yet to be fully elucidated, it does appear that the availability of SERT is a major requirement, and blocking SERT with fluoxetine protects against the toxic effects of MDMA (Sanchez *et al*., 2001; Shankaran *et al*., 1999). For neonatal rats to be less susceptible than adults to MDMA, when in the immediate post-natal period the density of SERT is so much higher than in adult, would therefore

Table 2 Local cerebral glucose utilization in brains of adult rats (P90) treated with either saline or MDMA *in utero* (E15–18) or postnatally (P10–13)

	In utero *treatment*		*Postnatal treatment*	
Brain region	*Saline*	*MDMA*	*Saline*	*MDMA*
Dorsal raphe	73±4	85±6	74±5	72±4
Median raphe	78±2	99±9	76±6	76±7
Locus coeruleus	51±2	70±3*	55±4	57±3
Substantia nigra				
Pars compacta	58±2	68±5	63±4	60±5
Pars reticulata	41±2	47±2	39±2	44±3
Inferior Olive	65±3	78±3*	65±2	60±5
Nucleus ambiguus	53±2	64±2*	58±3	56±3
Trigeminal nucleus	50±2	60±2*	53±4	57±4
Hippocampus				
Subiculum	63±3	81±6*	61±4	63±4
Dentate Gyrus	48±1	45±3	49±4	50±2
CA1	53±1	55±5	50±8	54±2
CA2	48±1	46±5	52±4	46±4
CA3	60±2	65±5	67±4	67±3
Neocortex				
Occipital	95±4	113±7	100±4	95±3
Parietal	85±3	100±9	82±4	89±4
Frontal	94±2	110±7	91±6	87±7
Cingulate	96±3	115±9	94±6	97±6
Thalamus				
Mediodorsal	88±6	100±6	84±6	88±5
Ventrolateral	63±3	70±3	60±6	64±2
Anterior	80±4	115±8*	84±6	86±3
Hypothalamus				
Medial	48±2	60±3*	45±3	45±5
Lateral	50±3	50±4	49±3	48±3
Septal nucleus	49±2	61±4*	48±2	52±4
Caudate nucleus	86±3	99±6	84±5	80±6
Globus pallidus	42±2	53±3*	40±3	38±4

Data are presented as mean local cerebral glucose utilization (μmol 100 g^{-1} min^{-1})±s.e.m. ($n=5$ in each group). *, Significantly different from control $P<0.05$; critical t for 8 d.f. = 2.306).

Table 3 Physiological status of adult animals used in the measurement of $lCMR_{glu}$

	In utero *treatment*	
Physiological variable	*Saline*	*MDMA*
pCO_2 (mmHg)	39.5±1.1	41.2±1.6
pO_2 (mmHg)	87.9±2.3	90.3±3.1
pH	7.41±0.03	7.40±0.02
Plasma glucose (mg l^{-1})	1.67±0.2	1.69±0.2
Corticosterone (nmol l^{-1})	71±3	73±4
MABP (mmHg)	130±5	126±4

Data are presented as mean±s.e.m. ($n=5$ in each group) and were measured immediately prior to the initiation of $lCMR_{glu}$.

appear to be counter-intuitive and might suggest that the distribution of [^{3}H]-paroxetine binding in the developing brain is not as closely related to 5-HT terminals as it is in adult. Certainly there is evidence of transient SERT expression in non-serotonergic primordial glutamatergic thalamo-cortical nerve terminals (Whitworth *et al*., 2002). It is also possible that levels of SERT expression change so that the ratio of [^{3}H]-paroxetine binding density to 5-HT terminals is higher than in adult. However, immunohistochemical studies clearly demonstrate that the spatial and temporal distribution of serotonin-immunoreactive axons in neonates matches exactly the pattern of functionally-mature neocortical SERT (D'Amato *et al*., 1987). It would appear reasonable therefore to assume that the density of [^{3}H]-paroxetine binding which we have observed in this study reflects, predominantly at least, serotonergic hyperinnervation and the dichotomy of MDMA resistance remains to be explained.

A non-specific cause of MDMA-resistance cannot be discounted. Small decreases in ambient temperature are known to markedly reduce MDMA-induced neurotoxicity (Malberg & Seiden, 1998) and the inevitable disturbance to the brooding activities of dams caused by the injection protocols, together with greater sensitivity to ambient temperature in neonates, could have had a neuroprotective effect. This could not however explain the lack of toxicity when the offspring were exposed to MDMA *in utero* and nor are there likely to be critical reductions in core temperature in animals post-weaning. A more specific explanation may be found in the changing morphology of developing 5-HT neurones. In the adult, serotonergic neurones form two distinct populations which can be differentiated on the basis of the morphological characteristics of their axons and terminals; thick, varicose fibres emanate predominantly from the median raphé nucleus and fine axons from the dorsal raphé nucleus (Kosofsky & Molliver, 1987). The fine 5-HT axon terminals prove to be extremely vulnerable to MDMA-induced neurotoxicity whilst the thicker, varicose fibres have been found to be relatively spared (O'Hearn *et al*., 1988). It is of particular interest therefore that the serotonergic fibres which contribute to the transient hyperinnervation of

neocortex during early postanatal life have been described as varicose (D'Amato *et al*., 1987), but these give way to finer axons by the time the animals reach adulthood (Molliver, 1987).

It is tempting to speculate that the coincidence of cortical maturation and the appearance of an adult pattern of SERT distribution, and possibly neuronal morphology, is causally linked with the onset of vulnerability to the neurotoxic properties of MDMA. However, it has also been suggested that the onset of a toxic response relates not to maturation of 5-HT terminals *per se*, but rather to the development of dopaminergic systems in the brain (Aguire *et al*., 1998), although the role of dopamine in the toxicity of MDMA in general has been seriously questioned (Colado *et al*., 1999). Alternatively, it is quite possible that during this highly labile phase of brain development MDMA is indeed neurotoxic, but that terminals quickly re-grow so that no deficit is evident at the time of measurement. In order to investigate this possibility, we treated neonatal rats on P10–P13 and sacrificed the animals at P25. If the 5-HT terminals had been disrupted by the MDMA, and had regrown, it might have been expected that the development of the adult pattern of cortical paroxetine binding might have been delayed. Although perhaps not providing the most definitive evidence that re-growth was not a factor in masking MDMA toxicity, the fact that the timing of cortical development was not delayed is at least suggestive.

Despite the apparent lack of evidence for any lasting effects of *in utero* exposure to MDMA upon serotonergic terminals in the rat brain, as determined by the density of SERT, there is clear evidence of increased $lCMR_{glu}$ in the locus coeruleus and those areas of the forebrain which receive noradrenergic projections from this nucleus. However, not all areas of the forebrain which receive ascending projections from the locus coeruleus are metabolically activated in these animals (e.g. neocortex). This effect was evident only when the rats were exposed *in utero*, and was not found in rats treated postnatally, suggesting that there might be a critical period in development at which effects are manifest. Previous behavioural studies have also suggested that there might be a critical period during which MDMA might induce long-term impairments in spatial learning and memory (Broening *et al*., 2001), but the period is much later than we have identified (between P11 and P20), with no discernible effects of earlier exposure being reported. Given the $lCMR_{glu}$ data presented here, it is possible that effects of prenatal MDMA might be found in a whole variety of behaviours in which noradrenergic systems are involved, including responsiveness to environmental cues (Lapiz *et al*., 2000) and defence behaviour (Neophytou *et al*., 2001), as well as the retention of spatial information (Lapiz *et al*., 2001). These are certainly areas which might be worth further study, but given the relatively circumscribed changes observed here, the effects on these behaviours might be expected to be rather subtle.

The effects of *in utero* exposure to MDMA in locus coeruleus could reflect a more generalized toxic (or teratogenic) action of MDMA which early in ontogeny might lack specificity for 5-HT neurones and have instead an extended action to involve monoaminergic neurones in general. Alternatively, locus coeruleus is known to receive 5-HT innervation (Leger & Decarries, 1978; McRae-Degueurce *et al*., 1982), and, in adults, brain stem nuclei are as susceptible as is forebrain to the toxic effects of MDMA (Harvey *et al*., 1993). It is possible, therefore, that in the medulla, physically close to the source of 5-HT projections, serotonergic innervation adopts the mature, MDMA-vulnerable form much earlier in development, allowing MDMA toxicity to be manifest. We are currently testing this hypothesis using quantitative radioligand autoradiography on serial sections through the medulla of mature rats treated *in utero* with MDMA.

Despite the apparent lack of evidence for MDMA-induced serotonergic terminal loss following perinatal exposure, this study suggests that exposure of the human foetus to MDMA is not without risk. Whatever the underlying mechanism might be, our results show that exposure to MDMA *in utero* results in relatively widespread changes in cerebral function which persist into adult life. The stage of development at which these rats were treated corresponds to early *in utero* life in man, and these studies therefore further highlight the potentially harmful effects of this drug, despite its widely perceived 'safety'.

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