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Early life adversity programs changes in central 5-HT neuronal function in adulthood.

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Abstract

Early life adversity is associated with an increased incidence of psychiatric illness in adulthood. Although the mechanisms underlying this association are unclear one possible substrate is brain 5-HT neurotransmission, which is reportedly abnormal in several psychiatric disorders. This study examined the effect of a rat model of early life adversity - early maternal separation (EMS) - on 5-HT neurotransmission in adulthood. *In vitro* electrophysiological experiments revealed that, in EMS rats compared to controls, the sensitivity of α_1 -adrenoceptors on 5-HT neurones in the dorsal raphe nucleus was significantly reduced whilst the sensitivity of 5-HT_{1A} receptors showed a non-significant trend to reduction. In *in vivo* microdialysis experiments the 5-HT_{1A} receptor agonist-induced suppression of 5-HT release in the frontal cortex was reduced in EMS animals suggesting desensitization of 5-HT_{1A} autoreceptors. There was no increase in basal 5-HT in the frontal cortex as measured by microdialysis and a non significant trend towards increased basal firing activity of classical (non-bursting) 5-HT neurones in the DRN measured by *in vivo* electrophysiology. Finally, EMS failed to alter expression of mRNAs coding for 5-HT_{1A} or α_{1B} receptors in the DRN as measured by *in situ* hybridisation histochemistry, suggesting that functional changes in receptor sensitivity observed are not due to changes in receptor gene transcription. The findings demonstrate that early life adversity programs changes in sensitivity of the two principal regulators of 5-HT neuronal activity. Similar effects in humans may contribute to the increased incidence of psychiatric illness in individuals exposed to early life adversity.

Introduction

In man the experience of adverse events early in life is associated with an increased risk of the development of psychiatric disorders in adulthood. There is an increased incidence of disorders including major depression, anxiety disorders and suicidality in adults who as children experienced adversity, such as instability of family relationships, parental loss, sexual or physical abuse and extreme poverty (Breier *et al.*, 1988; Agid *et al.*, 1999; Sadowski *et al.*, 1999; Weiss *et al.*, 1999). This association has lead to a widely held belief that the stress of early adverse experiences programs changes in the brain which persist throughout the lifetime and which predispose an individual to the development of psychopathology (for review see Heim & Nemeroff, 2001). There is much evidence to implicate dysfunction in central monoamine neurotransmission, in particular 5-HT neurotransmission, in a variety of psychiatric disorders, including major depression (Mann *et al.*, 1995; Drevets *et al.*, 2000; Bhagwagar *et al.*, 2002), anxiety (Graeff, 1997; Nutt, 2001) and suicidality (Arango *et al.*, 1995; Mann *et al.*, 2001). Given the association of psychiatric disorders with both early life adversity and 5-HT dysfunction, we reasoned that early life adversity might cause persistent changes in the regulation of 5-HT neurotransmission, and this may serve as a vulnerability factor in the development in adulthood of psychiatric disorders. Indeed, the development of 5-HT neuronal systems is incomplete at birth and marked developmental changes in structure and function continue during the pre-pubertal period (Lidov & Molliver, 1982; Ugrumov *et al.*, 1986; Smith & Gallagher, 1989; Boylan *et al.*, 2000). Furthermore, there is evidence that environmental and pharmacological manipulations applied during this pre-pubertal stage can cause profound changes in features of 5-HT neurotransmission, which persist into adulthood (Stachowiak *et al.*, 1984; Maudhuit *et al.*, 1995; Whitaker-Azmitia *et al.*, 2000).

Rat models of early life adversity include those in which the neonatal animals are periodically deprived of contact with the dam: usually known as early maternal separation (EMS). These models have been widely used to elucidate the functional and biochemical consequences of early life adversity and have revealed a number of persistent effects including changes in hypothalamo-pituitary-adrenal axis function (Plotsky & Meaney, 1993; Anisman *et al.*, 1998), anxiety-related and conditioned behaviors (Matthews *et al.*, 1996; Maciag *et al.*, 2002), and noradrenaline neurotransmission (Liu *et al.*, 2000). Recently EMS has been reported to induce changes in tissue concentrations of 5-HT in selective regions of the cortex and hippocampus (Matthews *et al.*, 2001), but to date no studies examining functional aspects of 5-HT neurotransmission in the adult have been reported. The present study examined the programming effects of EMS on features of central 5-HT function in the dorsal raphe nucleus (DRN)-frontal cortex ascending projections. *In vitro* electrophysiology, *in situ* hybridisation histochemistry, *in vivo* electrophysiology and microdialysis were used to examine the effects of EMS on the sensitivity and transcription of 5-HT_{1A} and α_1 adrenoceptors (the major inhibitory and excitatory drives on 5-HT neurones) and on net 5-HT neuronal function *in vivo*.

Materials and Methods

Animals and EMS procedure

All procedures were approved by the University of Newcastle ethical committee and were undertaken in accordance with the UK Animals (Scientific Procedures) Act 1986. Lister hooded rat pups were bred in-house from a stock of 12 breeding females (9 control mothers and 3 EMS mothers) and 4 males. Between postnatal days 1 and 3, litters were counted and, where necessary, reduced to 10 by removal of female pups. Throughout the pre-weaning period both EMS and control animals were handled briefly at three-day intervals to allow cage cleaning. EMS was carried out according to an established protocol (Matthews *et al.*, 1996) in which pups in litter groups were separated from their mothers for periods of 6 h on 10 occasions between postnatal days 5 and 20. During the separation pups were housed in a small polythene cage lined with tissue and containing nesting material taken from the home cage. The cage was placed in an incubator with base temperature set at 36°C, which was humidified to approximately 50% humidity. Separations took place during the light phase (lights on: 07.00-19.00). At the end of the separation period the pups, together with the nesting material, were returned to their mother. Control litters were left undisturbed. Following weaning at 21-23 days, the male EMS and control pups were housed in litter groups of 2-6 animals and did not undergo further interventions (aside from routine cage cleaning) until they reached 65-80 days of age, when they were weighed and tail marked for identification. Each data set contains animals from at least two litters. Some of the animals used for *in vitro* electrophysiology and *in situ* hybridisation histochemistry were exposed to an elevated plus maze for 10 min immediately before sacrifice (data not reported).

In vitro electrophysiology

Recordings were undertaken essentially as previously described (Lowry *et al.*, 2000). Control and EMS rats were sacrificed with an overdose of halothane and decapitated. The brain was immediately removed and coronal slices (400 μm) containing the DRN were cut on a vibratome and collected into oxygenated artificial cerebrospinal fluid (CSF; composition (mM): NaCl 124; KCl 3.25; MgSO_4 2.4; CaCl_2 2; KH_2PO_4 1.25; NaHCO_3 26; D-glucose 10; equilibrated with 95% O_2 /5% CO_2) at room temperature. Slices were transferred to a recording chamber maintained at 37°C and superfused with oxygenated artificial CSF (0.5 ml/min). After at least 1 h equilibration, the artificial CSF was supplemented with 1 μM phenylephrine (PE) to provide a constant α_1 -adrenergic stimulation. Extracellular recordings of spontaneous activity were made using glass electrodes filled with 2M NaCl and collected onto a PC using Spike 2 software (CED). Putative 5-HT neurones were identified on the basis of their location in the DRN and electrophysiological characteristics (VanderMaelen & Aghajanian, 1983). Between 3 and 7 neurones (in 1-3 slices) were recorded from each animal. Test drugs were applied for periods of 2 min by switching the artificial CSF to one containing the drug of interest. Neurones from 7 control animals (from 4 litters) and 8 EMS animals (from 3 litters) were used for determination of responses to 5-HT and PE. Neurones from a further 4 control animals (from 2 litters) and 4 EMS animals (from 2 litters) were tested for their responses to 5-HT only. Multiple concentrations of 5-HT (range: 10 - 300 μM) were tested in the presence of 1 μM PE in order to estimate an EC_{50} for suppression of firing. The response to PE at 3 and 6 μM in addition to the baseline concentration of 1 μM were also determined. Basal firing rate quoted is that measured at the start of the recording. For each drug response, the basal firing rate in a 120 s epoch immediately before the addition of the drug of interest was calculated. The responses to 5-HT and PE were determined in a

120 s period centred on the maximal inhibitory or excitatory effect of the drug. The firing rate in this period is expressed as a percentage decrease or increase relative to the baseline period. An EC₅₀ for 5-HT on each neurone was calculated by interpolation of concentration-percentage inhibition data on a semi-log plot.

In situ hybridization histochemistry

Eight control rats (from 5 litters) and 8 EMS rats (from 3 litters) were sacrificed by overdose of halothane and rapidly decapitated. The brain was immediately removed, frozen in isopentane cooled in liquid nitrogen, and stored at -70°C before sectioning. Sections (12 μm) of the midbrain were cut on a cryostat and thaw mounted onto RNAase-free gelatine-coated slides. Four sections per slide were collected approximately 120 μm apart. Sections were pre-treated as follows: initial fixation for 5 min in 4 % paraformaldehyde in phosphate buffered saline; two washes in phosphate buffered saline; acetylation for 10 min with 0.25 % acetic anhydride in 0.1 M triethanolamine buffer; dehydration through graded ethanol washes and defatting by a 10 min chloroform wash and treatment with 100 % and 95 % ethanol. Slides were air-dried and stored at -20°C . *In situ* hybridisation histochemistry was performed using oligonucleotide probes synthesised by the Molecular Biology Unit of Newcastle University. The probe for the 5-HT_{1A} receptor mRNA was complementary to bases 2105-2151 of the rat 5-HT_{1A} receptor gene (AF217200) (5' ggt tag cgt ggg agg aag gga gac tag ctg tct gag cga cat aca ag 3') and that for the α_{1B} receptor mRNA was complementary to bases 1420-1467 of the rat α_{1B} receptor (X51585) (5' tcg ctg gag aga tca cag tcg cgg aag gac tct ctg gat gac agc ggc 3') (Pieribone *et al.*, 1994). BLAST searches against all published RNA sequences was performed to confirm specificity of the probe. Oligonucleotide probes were 3' end labelled using [³⁵S]-ATP and terminal deoxynucleotidyl transferase (Amersham).

Labelled oligonucleotide was purified on a Sephadex G50 size-exclusion column and was added to a hybridization mixture (50 % formamide, 4 x standard saline citrate (SSC), 10 % dextran sulphate, 5 µl Denhardt's, 200 µg/ml salmon sperm DNA, 100 µg/ml poly A, 25 mM sodium phosphate, 1 mM sodium pyrophosphate and 5 % dithiothreitol). Hybridization mixture (200 µl containing approximately 2×10^6 cpm per slide) was applied to each slide. Slides were coverslipped and incubated overnight at 35°C. Coverslips and excess hybridization mixture were washed off in 1 x SSC, followed by three 20 min washes in 1 x SSC at 58°C and two 60 min washes in 1 x SSC at room temperature. Following a final rinse in deionized water slides were air dried, mounted onto card and apposed to Biomax film (Kodak Eastman) in an autoradiographic cassette along with [^{14}C] calibration strips (Amersham). Films were developed using an automated Agfa Curix compact plus daylight™ processor. Images from developed autoradiograms were captured on digital camera and analysis was performed using Scion Image software (NIH). The maximal optical density within the region of the DRN in each of four brain sections was determined and a mean value for each animal calculated. Optical density values were calibrated by reference to the [^{14}C] standards and are expressed in arbitrary units.

In vivo electrophysiology

Six control animals (from 2 litters) and 9 EMS animals (from 3 litters) were used for *in vivo* electrophysiological recording. Rats (body weight: 265-309 g) were anesthetized with chloral hydrate (500 mg/kg i.p.) and placed in a stereotaxic frame in the flat skull position (incisor bar -3.3 mm). The skull was exposed and a burr hole was drilled over the DRN in the midline (bregma -7.6 mm). A screw fixed into the right frontal bone served as the ground connection. A glass microelectrode filled with 2 M NaCl and 2 % Pontamine Sky Blue (4-8 MΩ *in vitro*

resistance) was lowered to just above the DRN (4.5 mm below the dura). Signals were amplified (x1000), filtered (300-1500 Hz band pass) and recorded on digital audio tape. The electrode was lowered slowly using a remote micromanipulator (Narashige) and all spontaneously active neurones encountered were recorded. Baseline recordings were of at least 4 min duration. After each recording the electrode was moved ventrally. Descents through the DRN were made in a systematic manner. In most cases the first descent yielded several closely spaced presumed 5-HT neurones in the dorsal part of the electrode track. Where this was the case, the descent was continued in this track to a total depth of 6.5 mm below dura. Further descents using the same electrode were then made within 200 μ m of the initial descent. In cases where few or no neurones were encountered in the dorsal part of the first descent, the electrode was raised and either a second electrode, or the same electrode, was lowered at a position adjacent to the first descent. Up to 25 neurones were recorded per animal. Data were analysed using Spike 2 software. Firing rates of the neurones were determined usually over a period of 160 s but occasionally over shorter periods when interference affected the recording. The original recordings were also examined for bursting activity (Hajós *et al.*, 1995).

In vivo microdialysis

Seven control animals (from 3 litters) and 8 EMS animals (from 2 litters) were used for *in vivo* microdialysis. Rats (body weight: 274-355 g) were anesthetized with chloral hydrate (500 mg/kg i.p.) and placed in a stereotaxic frame in the flat skull position (incisor bar –3.3 mm). The skull was exposed and a trephine hole drilled over the right frontal cortex (coordinates from bregma: anterior-posterior: +3.2 mm, medio-lateral: 3.0 mm). A small screw was fixed into left frontal bone. A concentric style microdialysis probe with 3.0-3.5 mm dialysing window (Hospal AN69 membrane) was implanted into the frontal cortex to a depth

of 4.6 mm below dura, and cemented to the skull and screw using dental acrylic. A lateral tail vein was cannulated using a 25-gauge hypodermic needle. The probe was continuously perfused at 2.3 $\mu\text{l}/\text{min}$ with an artificial CSF (composition (mM): NaCl 140; KCl 3; CaCl_2 2.4; MgCl_2 1.0; Na_2HPO_4 1.2; NaH_2PO_4 0.27; glucose 7.2; pH 7.4) containing the serotonin re-uptake inhibitor fluoxetine (10 μM). Dialysates collected over 20 min periods were stored on ice until assay by HPLC. 5-HT was separated from other dialysate components by means of reverse phase chromatography using a 100 mm x 4.6 mm Microsorb (3 μm C18) column (Varian) in a column oven set at 30°C. The mobile phase (15 % methanol; 127 mM NaH_2PO_4 ; 25 μM octane sulphonic acid; 0.045 % EDTA; pH 3.9) was pre-oxidised on a guard cell set at 250mV. 5-HT was oxidised on a coulometric sensor comprising two electrochemical cells with potential differences set at 60 mV and 200mV (ESA). The resultant current change on cell 2 was detected using a Coulochem detector (ESA) and was quantified and displayed on an integrator (Waters). The content of 5-HT in each dialysate was determined by the peak height with reference to an external 5-HT standard assayed daily. Following the establishment of a stable baseline (2-3 h post-probe implantation), animals were injected with the 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT) dissolved in water and administered i.v. (1 ml/kg body weight). The initial dose of 3 $\mu\text{g}/\text{kg}$ was followed 60 min later by 30 $\mu\text{g}/\text{kg}$ and, finally, a further 60 min later by 300 $\mu\text{g}/\text{kg}$. Dialysates were collected until 1 h after the final dose of 8-OHDPAT. Probe placement was verified by *post-hoc* inspection of brain sections.

Statistical analysis

Data from individual neurones recorded in the *in vitro* and *in vivo* electrophysiological experiments are presented. For statistical analysis, data from individual neurones were

grouped by animal such that n= number of animals in each group. Differences between group means were analysed by independent t-test or two-way ANOVA following test for normality using Kolmogorov-Smirnov test. *Post-hoc* analysis was by Tukey test. Significance at the $p<0.05$ level is reported.

Results

Effect of EMS on α_1 adrenoceptor and 5-HT_{1A} receptor-mediated regulation of 5-HT neuronal activity in vitro

Putative 5-HT neurones were recorded from DRN slices from control and EMS animals. All of the neurones recorded (n=99) displayed electrophysiological characteristics of 5-HT neurones - regular discharge of positive-negative-positive action potentials (VanderMaelen & Aghajanian, 1983; Jacobs & Fornal, 1991) - and fired between 0.4 and 5.5 spikes/s in 1 μ M PE. For all neurones tested, application of 5-HT caused an inhibitory response that was dose-dependent and rapidly reversed upon drug withdrawal (Fig. 1). Figure 2 shows the EC₅₀ values for individual neurones recorded from slices taken from EMS and control animals together with the mean EC₅₀ grouped by animal. The EC₅₀ for this inhibitory response calculated from the average value per animal showed that there was no significant difference between groups in the sensitivity to 5-HT (Fig. 2 bars; EC₅₀: 23.0 ± 3.0 μ M (n = 11) vs. 35.0 ± 6.2 μ M (n = 12), control vs. EMS, p = 0.1, t-test). However, examination of the distribution of EC₅₀ values for individual neurones revealed a much greater variation in EC₅₀ in neurones from EMS animals with a greater incidence of neurones displaying insensitivity to 5-HT in the EMS animals than in the controls (Fig. 2 individual points).

The sensitivity of DRN neurones to PE was examined in 11 control and 12 EMS animals. Basal firing rate (i.e. that determined in the presence of 1 μ M PE) did not differ between groups (Fig 3 bars) nor was there any marked difference in intra-animal variability in firing rates between EMS and control animals (Fig. 3, individual points). In 58 neurones from 7 control and 8 EMS animals, responses to further concentrations of PE were determined. Raising the concentration of PE from baseline (1 μ M) to 3 or 6 μ M caused concentration-

related excitatory responses, which readily reversed (Fig. 1). The response to PE was significantly attenuated in the EMS animals when compared to the controls (Fig. 4). Two-way ANOVA revealed a significant effect of the PE concentration ($F_{2, 26} = 86.7$, $p < 0.001$) and a significant treatment x concentration interaction ($F_{2, 26} = 6.0$, $p < 0.01$). *Post-hoc* Tukey test revealed that excitation was significantly reduced in response to 6 μ M PE in EMS treated animals $p < 0.05$.

Effect of EMS on the expression of mRNAs encoding 5-HT_{1A} and α_{1B} receptors in the DRN

Both 5-HT_{1A} and α_{1B} receptor mRNAs were densely expressed in the region of the DRN with relatively low expression elsewhere in the midbrain (Fig 5). Quantitative densitometry of the autoradiograms revealed that there was no difference between EMS and control animals with respect to the expression of both 5-HT_{1A} mRNA (optical density (arbitrary units): control: 9.28 ± 0.15 ($n = 7$) vs. EMS: 8.92 ± 0.12 ($n = 8$)) and α_{1B} mRNA (optical density (arbitrary units): control: 34.87 ± 0.82 ($n = 7$) vs. EMS: 35.65 ± 1.28 ($n = 6$)).

Effect of EMS on 5-HT neuronal function in vivo

Recordings were made of spontaneously active DRN neurones in anesthetized animals. Putative 5-HT neurones were sub-divided into ‘classical’ or ‘bursting’ on the basis of their firing rate and pattern according to published criteria (Vandermaelen & Aghajanian, 1983, Hajós *et al.*, 1995; Hajós & Sharp, 1996). Similar ratios of classical to bursting 5-HT neurones were found in the two groups (ratio of classical to bursting: 62 to 11 ($n=83$ neurones) (75 to 25 %) in control animals vs. 83 to 28 ($n=111$ neurones) (75 to 25 %) in EMS animals). The mean (grouped by animal) basal firing rate of classical neurones in the EMS and control groups was not significantly different (mean $\pm$ SEM: 8.9 ± 0.9 ($n = 6$) vs. $12.3 \pm$

1.8 spikes/10 s ($n = 9$), control *vs.* EMS; $p = 0.12$; Fig. 6, bars). However, there was a wide variation in firing rates in these classical 5-HT neurones, and the incidence of neurones with higher basal firing rate appeared to be much greater in the EMS group compared to the controls (Fig. 6, individual points). When the burst firing neurones were grouped by animal, EMS and control groups did not differ with respect to the basal firing rate of these neurones (group means 10.5 ± 0.9 ($n = 4$) *vs.* 12.4 ± 2.9 ($n = 7$) spikes/10 s). There was also no difference in the distribution of firing rates between the two treatment groups (data not shown).

Effect of EMS on extracellular 5-HT in the frontal cortex

Basal dialysate 5-HT levels did not differ between control animals and EMS animals when measured in the presence of the selective 5-HT re-uptake inhibitor fluoxetine (44.0 ± 3.2 *vs.* 40.1 ± 3.0 fmol/sample, control *vs.* EMS ($p > 0.05$)). Administration of the 5-HT_{1A} receptor agonist 8-OHDPAT (3-300 $\mu\text{g/kg}$ i.v.) caused a dose-dependent decrease in dialysate 5-HT levels (Fig. 7). In EMS animals this response was significantly attenuated compared to control animals, an effect which was most evident following the two higher doses of 8-OHDPAT (Fig. 7). Two-way ANOVA (% data from +20 min) revealed significant main effects of time ($F_{8, 104} = 149.0$, $p < 0.001$) and treatment ($F_{1,13} = 6.1$, $p < 0.05$) as well as a significant time x treatment interaction ($F_{8, 104} = 6.3$, $p < 0.01$).

Discussion

Much evidence associates the development of psychiatric disorders in adulthood with both early life adversity and dysfunction of central 5-HT systems. It is hypothesised that early life adversity programs long-term changes in 5-HT neurotransmission, which in turn act as a vulnerability factor for psychiatric disorders. To examine this hypothesis, the impact of a rat model of early life adversity on aspects of central 5-HT function in adulthood was determined. EMS significantly decreased the sensitivity of α_1 receptors mediating excitation of DRN 5-HT neurones and 5-HT_{1A} receptors regulating 5-HT release in the frontal cortex. These changes in receptor sensitivity were not accompanied by changes in receptor mRNA expression. Thus, the present data show that early life adversity programs persistent changes in the sensitivity of 5-HT neurones to both excitatory and inhibitory regulators of 5-HT neurotransmission in adult animals.

The present *in vitro* electrophysiological experiments demonstrated that excitatory responses of DRN 5-HT neurones to application of PE were diminished in slices taken from EMS rats compared to controls. The firing activity of 5-HT neurones *in vivo* is under excitatory regulation by α_{1B} receptors located on 5-HT neuronal perikarya (Gallager & Aghajanian, 1976; Pieribone *et al.*, 1994), and *in vitro* recordings of DRN neurones are usually made in the presence of the α_1 -adrenoceptor agonist, PE, in order to evoke 'spontaneous' activity in the otherwise silent neurones (e.g. Sprouse *et al.*, 1990; VanderMaelen & Braselton, 1990; Corradetti *et al.*, 1998). A relatively low concentration of PE was present for all basal recordings and the concentration was increased to examine the sensitivity of the α_1 -adrenoceptor. The data show that the excitatory response to PE was attenuated in EMS animals compared to controls. This subsensitivity was evident across the whole population of

5-HT neurones recorded, and is suggestive of a desensitisation of α_{1B} receptors on DRN 5-HT neurones.

We also tested the sensitivity of 5-HT neurones to 5-HT itself. Evidence suggests that the autoinhibitory response to 5-HT is mediated by the somatodendritic 5-HT_{1A} receptor since it is completely blocked by the selective 5-HT_{1A} receptor antagonist, WAY 100635 (Johnson *et al.*, 2002). Although we found that there was no significant effect of EMS overall on 5-HT sensitivity, there was evidence of decreased sensitivity to 5-HT in a subpopulation of neurones in EMS rats. Thus, a greater proportion of neurones in the EMS rats had high EC₅₀ for 5-HT. A reduced sensitivity to 5-HT is consistent with desensitization of somatodendritic 5-HT_{1A} receptors in this population of neurones.

In order to determine aggregate (i.e. population) effects of EMS on 5-HT_{1A} autoreceptor sensitivity, we used *in vivo* microdialysis to measure the decrease in extracellular 5-HT in the frontal cortex in response to administration of the 5-HT_{1A} receptor agonist 8-OHDPAT. The frontal cortex is an area that is innervated by 5-HT neurones projecting from the DRN (McQuade & Sharp, 1997). Consistent with previous studies (Sharp *et al.*, 1989; Hjorth & Sharp 1993), administration of the 5-HT_{1A} receptor agonist 8-OHDPAT decreased 5-HT release. The effect of systemically administered 8-OHDPAT on terminal 5-HT release is mediated (at least in part) through activation of the 5-HT_{1A} autoreceptors in the DRN (Hutson *et al.*, 1989), and the attenuated effect of 8-OHDPAT seen in EMS animals would be consistent with a desensitization of DRN 5-HT_{1A} autoreceptors.

Our *in vivo* microdialysis data are not completely consistent with our *in vitro* electrophysiological finding of no overall reduction in population 5-HT_{1A} sensitivity.

However, several possible explanations for this apparent discrepancy present themselves: first, it is possible that those individual neurones recorded *in vitro* and found to have low 5-HT_{1A} receptor sensitivity represent the population of neurones which project to the frontal cortex. Second, it may be that desensitization of 5-HT_{1A} receptors in only a small percentage of neurones is sufficient to cause a marked change in 5-HT_{1A} receptor mediated suppression of 5-HT release in terminal regions. Finally, it should be noted that 8-OHDPAT when given systemically will also activate 5-HT_{1A} receptors in the forebrain. 5-HT_{1A} receptors on medial prefrontal cortical neurones have been shown to contribute to the inhibitory effect of systemic 8-OHDPAT on 5-HT neuronal firing and release *in vivo* (Ceci *et al.*, 1994; Hajós *et al.*, 1999). Thus, the effect of EMS on the response to 8-OHDPAT may reflect desensitisation of postsynaptic 5-HT_{1A} receptors as well those on 5-HT neurones in the DRN.

In view of the observed differences in 5-HT_{1A} and α_{1B} receptor-mediated responses, the effect of EMS on the expression of mRNAs encoding these two receptor subtypes was examined in the DRN. Both 5-HT_{1A} and α_{1B} receptor mRNAs were densely expressed in the DRN as has been previously described (Pompeiano *et al.*, 1992; Nicholas *et al.*, 1996). However, there were no differences in densities of expression between EMS and control animals suggesting that the *in vitro* electrophysiological and *in vivo* microdialysis findings are unlikely to be explained by changes in receptor gene transcription and a consequent change in receptor protein. It is possible that the lack of change in 5-HT_{1A} receptor mRNA observed is a function of the methodology which may not be sufficiently sensitive to a change in only a subpopulation of neurones. However, it is worthy of note that environmental, hormonal, and pharmacological manipulations have previously been reported to cause functional desensitization of 5-HT_{1A} receptors without a concomitant change in 5-HT_{1A} receptor binding (Laaris *et al.*, 1995; Lanfumey *et al.*, 1999; Hensler, 2002). These findings suggest that post-

receptor components such as G protein coupling and/or second messenger systems may underlie the reduced function. Similarly changes in post-receptor elements of the α_1 adrenoceptor may underlie the desensitization observed here.

Whilst our *in vitro* electrophysiological and *in vivo* microdialysis studies revealed evidence of desensitisation of both α_1 and 5-HT_{1A} receptors on 5-HT neurones in the DRN, there was little evidence of these changes causing alterations in basal 5-HT neurotransmission measured in anaesthetized rats *in vivo*. Thus using *in vivo* electrophysiology we found that proportions of classical and burst firing neurones did not differ between groups, and that there was no difference in the basal firing of either group of neurones. However, it is perhaps of interest that in the EMS animals the range of firing rates within individual animals was somewhat increased, with a greater proportion of neurones having relatively high basal firing activity. These data perhaps suggest that EMS causes an increase in the spontaneous *in vivo* activity of a sub-population of classical (non-bursting) DRN 5-HT neurones. Increased firing rate would be consistent with increased α_1 -adrenoceptor mediated excitation or decreased 5-HT_{1A} receptor inhibition and/or changes in tonic regulation of these neurones by other neurotransmitters (Baraban & Aghajanian, 1980; Piñeyro & Blier, 1999; Abellan *et al.*, 2000). However, in spite of any possible increased activity of a proportion of 5-HT neurones, it is of note that in our *in vivo* microdialysis study we found no increase in basal 5-HT levels in the frontal cortex. Since the perfusion medium contained the selective 5-HT re-uptake inhibitor fluoxetine, changes in dialysate 5-HT can be considered to reflect changes in release rather than alterations in 5-HT re-uptake. The lack of difference in basal dialysate 5-HT between the two groups of animals, suggests that the increased firing rate of the sub-population of neurones recorded *in vivo* was insufficient to influence the aggregate 5-HT release in the cortex. However, a caveat to our conclusion that basal 5-HT neurotransmission is unaltered

by EMS is that all measurements were made in anaesthetized animals. It is possible that under other conditions, the influences of changes in α_1 and 5-HT_{1A} receptor sensitivity would be revealed.

Although the mechanisms underlying the programmed changes following EMS are not the subject of the present study, it is established that brain 5-HT systems continue to develop during the early postnatal period (Lidov and Molliver, 1982; Ugrumov et al., 1986; Boylan et al., 2000) and it is possible that the stress of EMS modulates this developmental process. However, it is perhaps of note that Smith & Gallager (1989) reported that α_1 and 5-HT_{1A} receptor sensitivities in the DRN do not change over the preweaning postnatal period in normally reared rats. Interestingly several groups have shown that 5-HT_{1A} (Joëls et al., 1991; Laaris et al., 1995; Leitch et al., 2003) and α_1 (authors' unpublished observations) receptor sensitivities in the DRN can be modulated by corticosterone. Although in the present study we did not examine circulating corticosterone, other workers using similar paradigms of EMS consistently report hypothalamo-pituitary-adrenal axis hyperactivity in adult rats subject to separation (Plotsky & Meaney 1993; Patchev et al., 1997; Anisman et al., 1998). Hence it is possible that persistent changes in the hypothalamo-pituitary-adrenal axis may underlie the changes in regulation of 5-HT neurotransmission. Further studies are underway to address this issue.

The present data show that EMS (a model of early life adversity) programs persistent changes in the sensitivity of 5-HT neurones to both excitatory (α_1) and inhibitory (5-HT_{1A}) regulators of 5-HT neurotransmission in adult animals. These data represent the first demonstration that functional alterations in 5-HT systems are induced by early life adversity. Although basal

5-HT neurotransmission measured *in vivo* was largely unaffected, the observed changes in 5-HT_{1A} and α_1 receptor sensitivity may result in changes in 5-HT transmission under specific conditions or changes in the adaptive responses to, for example, chronic stress. It is established that individuals who have suffered adversity early in life are at increased risk of the development of psychiatric disorders in adulthood (Breier *et al.*, 1988; Agid *et al.*, 1999; Sadowski *et al.*, 1999) and furthermore, evidence indicates that adults with psychiatric disorders have abnormalities in aspects of 5-HT neurotransmission (Arango *et al.*, 1995; Mann *et al.*, 1995; 2001; Nutt, 2001; Bhagwagar *et al.*, 2002). The important effects of EMS on the regulation of 5-HT function observed in the present study may, in part, explain the relationship between early life adversity and a lifelong increase in psychiatric morbidity observed in humans.

Abbreviations

ANOVA analysis of variance, ATP adenosine triphosphate, CSF cerebrospinal fluid, DNA deoxyribonucleic acid, DRN dorsal raphe nucleus, EC₅₀ 50% effective concentration, EDTA ethylenediaminetetracetic acid, EMS early maternal separation, HPLC high performance liquid chromatography, mRNA messenger ribonucleic acid, PE phenylephrine, SSC standard saline citrate, 5-HT 5-hydroxytryptamine, 8-OHDPAT 8-hydroxy-2-(di-n-dipropylamino)tetralin.

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Fig. 1. Typical ratemeter recording of firing activity in a putative 5-HT neuron in a DRN slice from a control animal. Record shows stable basal firing (in the presence of 1 μ M PE), inhibitory responses to 5-HT (25, 50 and 10 μ M), and excitatory responses to higher doses of PE (3 and 6 μ M). Bars indicate the period of application of drugs (2 min). Note that the records have been corrected for the lag time of the perfusion system.

Fig. 2. EC_{50} for autoinhibitory response of 5-HT neurons to exogenous 5-HT in DRN slices obtained from control and EMS animals. Vertical lines represent data from individual animals, with symbols representing EC_{50} values for individual neurons. Note that there is a much greater variability in the data from EMS animals with a greater number of insensitive neurons in control slices. Bars show mean (+SEM) EC_{50} value for control (open bar, $n=11$) and EMS (shaded bar, $n=12$) animals calculated from the average value for each animal.

Fig. 3. Basal firing rates of 5-HT neurons recorded *in vitro* in the presence of 1 μ M PE in DRN slices obtained from control and EMS animals. Vertical lines represent data from individual animals, with symbols representing firing rates of individual neurons. Bars show mean data (+ SEM) for basal firing rate in control (open bar; n=11) and EMS (shaded bar; n=12) animals calculated from the average value for each animal.

Fig. 4. Excitatory effects of the α_1 -adrenergic agonist PE on 5-HT neurons in DRN slices obtained from control animals (open symbols; $n=7$) and EMS animals (closed symbols; $n=8$). Data are the mean ($\pm$ SEM) firing rate under basal conditions (i.e. in the presence of 1 μ M PE) and following the increase of the concentration of PE to 3 and 6 μ M (semi-log plot). * $p<0.05$ *post-hoc* Tukey test.

Fig. 5. Autoradiograms showing expression of mRNAs encoding the α_{1B} adrenoceptor (A) and the 5-HT_{1A} autoreceptor (B) in the DRN. Within the midbrain, expression of both mRNA species was largely restricted to the DRN (large arrows) immediately below the aqueduct (small arrows).

Fig. 6. *In vivo* basal firing rate of DRN 5-HT neurons in rats subject to EMS and controls. Classical (non-bursting) 5-HT neurons (5-18 neurons / animal) were recorded from the DRN of chloral hydrate anesthetized rats. Vertical lines represent data from individual animals, with symbols representing basal firing rate for individual neurons. Note the greater number of individual neurones in the EMS animals with high basal firing rate. Bars show mean + SEM firing rates in control (open bar; n=6) and EMS animals (shaded bar; n=9).

Fig. 7. Effect of the 5-HT_{1A} agonist, 8-OHDPAT, on 5-HT release in the frontal cortex measured by microdialysis in chloral hydrate anesthetized rats. Data are mean $\pm$ SEM as percentage of the baseline (100 %) in control (open symbol; n=7) and EMS animals (closed symbol; n=8). Cumulative doses of 8-OHDPAT (3, 30 and 300 μ g/kg) were injected i.v. at hourly intervals as indicated by the arrows and hatched bars. Two-way ANOVA (% data from +20 min) revealed significant main effects of time ($F_{8, 104} = 149.0$, $p < 0.001$) and treatment ($F_{1,13} = 6.1$, $p < 0.05$) as well as a significant time $\times$ treatment interaction ($F_{8, 104} = 6.3$, $p < 0.01$). * $p < 0.05$ vs. respective value in control animals, *post-hoc* Tukey test.