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Prenatal Exposure to Fluoxetine (Prozac) Produces Site-Specific and Age-Dependent Alterations in Brain Serotonin Transporters in Rat Progeny: Evidence from Autoradiographic Studies[1]

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ABSTRACT

The present study provides the first autoradiographic evidence of age-dependent regional changes in the density of serotonin (5-HT) transporters in offspring following prenatal exposure to fluoxetine. Pregnant rats received either saline or fluoxetine (10 mg/kg, s.c.) daily from gestational day 13 through 20. The density of [^{3}H]citalopram-labeled 5-HT transporters was determined in forebrain regions and in midbrain raphe nuclei of prepubescent and adult male offspring. Brain regions representing integral components of the limbic system were particularly sensitive to the prenatal treatment. For example, prenatal fluoxetine exposure significantly altered the density of 5-HT transporters in subregions of the hypothalamus (dorsomedial nucleus, −21%; lateral hypothalamus, +21%), hippocampus (CA2, +47%; CA3, +38%), and amygdala (basolateral nucleus, +32%; medial nucleus, +44%) in prepubescent offspring. However, 5-HT transporter density in the dorsal and median raphe was unaltered in this same group of offspring. In adult offspring, 5-HT transporter densities, in all brain regions examined, were not significantly altered by prenatal exposure to fluoxetine. The present study also identifies significant age-related differences in 5-HT transporter densities between prepubescent and adult control offspring. For example, in adult control offspring, densities of 5-HT transporters were significantly greater in the cingulate cortex (+33%), basolateral amygdala (+58%), and CA1 area of the hippocampus (+78%); but significantly lower in the temporal cortex (−65%) and median raphe (−25%). The age-dependent and site-specific alterations in the density of 5-HT transporters suggests that either 5-HT innervation and/or 5-HT neuron function in various forebrain regions may be altered by prenatal exposure to fluoxetine.

Fluoxetine selectively inhibits the reuptake of serotonin (5-HT) into presynaptic nerve terminals thereby increasing synaptic concentrations of 5-HT (Fuller *et al.*, 1991; Thomas *et al.*, 1987). Repeated administration of fluoxetine to adult male rats has been demonstrated to markedly alter serotonergic neurotransmission as a result of fluoxetine-induced changes in brain 5-HT metabolism and 5-HT receptor density and function (Caccia *et al.*, 1992; De Montigny *et al.*, 1990; Li *et al.*, 1993; Welner *et al.*, 1989; Wong and Bymaster, 1981). Indeed, the therapeutic efficacy of fluoxetine is believed to be mediated by the drug's ability to enhance serotonergic neurotransmission upon repeated administration (Goodwin, 1996; Owens, 1996, 1997). Due to its relative selectivity, efficacy and safety in humans, (Fuller *et al.*, 1991; Thomas *et al.*, 1987; Wong *et al.*, 1991) fluoxetine is routinely prescribed to millions of Americans each year, including women of reproductive age, for the treatment of a variety of psychiatric disorders (Dubovsky, 1994; Hudson *et al.*, 1996; Kessler *et al.*, 1993; Sheehan and Harnett-Sheehan, 1996). Consequently, many women may continue to be treated with fluoxetine during pregnancy (Chambers *et al.*, 1996; Pastuszak *et al.*, 1993; Nulman *et al.*, 1997), increasing the potential for exposure of human offspring to fluoxetine. Yet, to date, few published studies have investigated the neurochemical teratogenic potential of this widely prescribed medication.

In humans, the research to date suggests that use of fluoxetine during embryogenesis neither increases the risk of fetal malformations nor produces behavioral abnormalities in preschool age children (Nulman and Koren, 1996; Nulman *et al.*, 1997; Pastuszak *et al.*, 1993). However, one study reports contradictory findings following drug exposure throughout the entire term of pregnancy (Chambers *et al.*,

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ABBREVIATIONS: ANOVA, analysis of variance; 5-HT, serotonin; PCA, *p*-chloroamphetamine; PD, postnatal day; SSRI, selective serotonin reuptake inhibitor.

1996). In general, the findings in humans indicating a lack of effect on offspring vital parameters, physical appearance, or behavioral measures following prenatal exposure to fluoxetine are supported by data from animal studies (Byrd and Markham, 1994; Cabrera and Battaglia, 1994; Hoyt *et al.*, 1989; Vorhees *et al.*, 1994). However, in contrast to the absence of obvious physical or behavioral anomalies following prenatal fluoxetine exposure, other evidence in rats indicates that prenatal exposure to fluoxetine can produce biochemical alterations in brain 5-HT systems in both immature and adult offspring (Cabrera *et al.*, 1994; Cabrera-Vera *et al.*, 1997; Montero *et al.*, 1990; Romero *et al.*, 1994). These studies are consistent with data demonstrating that both fluoxetine and its active metabolite, norfluoxetine, cross the placenta and enter fetal brain tissue (Pohland *et al.*, 1989). After infiltrating fetal brain tissue, fluoxetine may alter extracellular concentrations of 5-HT by interacting with 5-HT transporters present on developing neurons and glia. Recent studies indicate that 5-HT exerts a trophic influence on the outgrowth and targeting of 5-HT neuronal projections and on the maturation of 5-HT target tissues (Lauder, 1990; Whitaker-Azmitia *et al.*, 1996). Therefore, our hypothesis was that the disruption of 5-HT systems during fetal brain development by the administration of fluoxetine would result in biochemical alterations in brain 5-HT pathways in offspring.

We previously reported that prenatal exposure to fluoxetine, at the same dose used in this study, produces site-specific alterations in brain 5-HT content (Cabrera-Vera *et al.*, 1997), 5-$HT_{2A/2C}$ receptor density, and 5-$HT_{2A/2C}$ receptor-mediated hormone secretion in the absence of visually apparent physical abnormalities (Cabrera and Battaglia, 1994). The purpose of the present study was to determine whether prenatal exposure to fluoxetine would alter serotonergic innervation of various brain regions, as assessed from densities of 5-HT transporters (Battaglia *et al.*, 1987; Descarries *et al.*, 1995). *In vitro* autoradiography was employed to determine changes in 5-HT transporters in specific neuroanatomic regions, as our previous data indicated that alterations in brain 5-HT systems may be subtle and localized to discrete neuroanatomic loci (Cabrera-Vera *et al.*, 1997). In addition, since our previous research indicated that prenatal exposure to fluoxetine can alter brain 5-HT pathways in an age-specific manner (Cabrera-Vera *et al.*, 1997; Cabrera and Battaglia, 1994), the present study determined the density of 5-HT transporters at both prepubescent and adult ages. The data reported herein provide additional biochemical evidence that prenatal exposure to fluoxetine can alter select serotonergic pathways in offspring, and that these changes are age-dependent and discretely localized to specific neuroanatomic loci.

Methods

Animals. Pregnant Sprague-Dawley rats weighing 280–320 g were obtained from Zivic Miller (Zelienople, PA) and maintained in a temperature (22–24°C), humidity (50–55%)- and illumination (12:12 hr light/dark cycle, lights on at 7 a.m.)-controlled facility. The determination of gestational day zero was carried out by the supplier, and was defined by the presence of a copulatory plug. All procedures were conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health.

Dams. Gravid rats arrived in the laboratory on gestational day 5. Starting on gestational day 8 (GD 8), and continuing throughout the injection period, all experimental animals were placed on a nutritionally balanced liquid diet (Riley *et al.*, 1979). Experimental dams received injections of either 0.9% saline (2 ml/kg, s.c.), or fluoxetine hydrochloride (10 mg/kg/2 ml, s.c.) once daily (at 9 a.m.) beginning on GD 13, and ending on GD 20. After termination of the injection paradigm, all animals had free access to food and water. This exposure period (E13–20) represents a gestational time during which 5-HT neurons are rapidly differentiating, proliferating and sending out axonal projections to their respective target regions (Aitken and Tork, 1988; Lidov and Molliver, 1982). Therefore, the rapid development of the 5-HT system from GD 13 through birth makes this period of development particularly sensitive to perturbations in the serotonergic system.

We have previously reported (Cabrera and Battaglia, 1994) that this treatment paradigm does not alter either maternal weight gain or nutritional intake of the pregnant dams. In addition, 10 mg/kg fluoxetine administered once daily represents a treatment paradigm commonly utilized to assess the effects of repeated fluoxetine administration on adult animals (Li *et al.*, 1993).

Offspring. At birth (*i.e.,* postnatal day 0, PD 0), all offspring from each of the experimental groups were fostered to untreated, lactating dams in order to eliminate the possible influence of drug-induced differences in nurturing. At the time of fostering, pups (from dams of 10 or more pups per litter), were culled to 9 pups per litter (5 males, 4 females) until weaning. As we previously reported in detail using an identical treatment paradigm (Cabrera and Battaglia, 1994), fluoxetine exposed offspring do not exhibit any visually apparent physical abnormalities. Furthermore, there were no significant differences in fetal viability between control and fluoxetine-exposed litters (Cabrera and Battaglia, 1994). All pups were weaned on PD 21. The number of samples (N) within each group was comprised of single pups obtained from different litters. At the time of weaning, males were housed in groups of 2 or 3 rats per cage, and had free access to food and water. The density of serotonin uptake sites was determined in male progeny at either PD 28 or PD 70; time points representing pre- and postpubescent ages, respectively. The rationale to focus the present studies to male offspring was 2-fold: (1) for comparison with other studies investigating the effects of comparable doses of fluoxetine administered to adult male rats, and (2) to preclude the potential confounding influence of differing ovarian hormone levels on transporter densities in adult female offspring which may not be cycling in syncrony. In addition, these postnatal ages were chosen based on our previous work indicating that prenatal exposure to fluoxetine produces differential changes in pre- and postsynaptic components of 5-HT systems at adult (PD 70) *vs.* prepubescent (PD 28) ages (Cabrera and Battaglia, 1994; Cabrera-Vera *et al.*, 1997). Therefore, as the effects of fluoxetine exposure on brain 5-HT systems have repeatedly demonstrated age-dependent specificity, the current study examined the same two developmental ages to determine whether prenatal fluoxetine exposure also produces age-dependent changes in brain 5-HT transporters.

Autoradiographic localization of 5-HT transporters. Rats were sacrificed by decapitation, their brains were quickly removed, frozen on powdered dry ice, secured with parafilm and plastic wrap then stored at −70°C. Coronal sections of brains were obtained at −20°C using a cryostat (Hacker Instruments, Inc.) set to obtain sections 15 μm thick. Sections were thaw-mounted onto chrome alum/gelatin-coated microscope slides, and stored at −20°C until used for autoradiographic measurement of 5-HT transporters. Coronal sections were taken at the following 8 levels according to the rat atlas by Paxinos and Watson (1986): Bregma +3.70 mm, +1.00 mm, −0.30 mm, −1.80 mm, −2.80 mm, −3.14 mm, −4.80 mm and −8.00 mm.

Prior to the *in vitro* autoradiographic assay, slide-mounted brain sections were brought to room temperature. Autoradiographic localization of 5-HT transporters was determined according to a modifi-

cation of the protocol by D'Amato *et al.* (1987). Slide mounted sections were preincubated at room temperature for 15 min in 50 mM Tris HCl containing 120 mM NaCl and 5 mM KCl (pH 7.7 at 25°C). Sections were then incubated at room temperature in 0.7 nM [^{3}H]citalopram (specific activity 81 Ci/mmol; K_D = 0.84 nM) in the absence or presence of 1 μM paroxetine to determine nonspecific binding. Following incubation, sections were washed twice in ice cold 50 mM Tris HCl containing 120 mM NaCl and 5 mM KCl (pH 7.7 at 25°C) for 10 min each time then dipped in ice cold deionized water, and dried rapidly under a stream of cold dry air. Following the *in vitro* labeling of 5-HT transporters, the slides were dried overnight in slide boxes containing desiccant prior to apposing them to tritium sensitive film (Hyperfilm-^{3}H; Amersham Corporation, Arlington Heights, IL) at 4°C for 30 days to generate the autoradiograms. Slides containing low and high tritium standards (Amersham Corporation) were included with each film to control for exposure differences between films. The films were developed by placing them in Kodak Developer D-19 for 5 min, then in Kodak Indicator Stop Bath solution for 30 sec, followed by 5 min in Kodak Fixer. The films were subsequently placed in a running water bath containing Kodak Photo-Flo 200 Solution for 15 min, then air dried. Autoradiograms were quantitated on a Macintosh Quadra 950 computer using the public domain NIH Image program version 1.55 (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov). Standard curves of film grey scale values and radioactivity generated by tritium standards coexposed with labeled slides, was best fit by an exponential function. Grey scale values were converted to units of radioactivity and then to fmol of sites/mg tissue equivalent taking into account the specific activity of the radioligand.

Drugs. [^{3}H]Citalopram (81 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Paroxetine was a generous gift from SmithKline Beecham Pharmaceuticals (Philadelphia, PA). Fluoxetine was a generous gift from Eli Lilly and Co. (Indianapolis, IN). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Statistics. The data are represented as the group means and the S.E.M. Statistical analysis of the data was performed by two-way analysis of variance (ANOVA). If the F values from the ANOVA indicated significant differences, individual group means were then compared by Newman-Keuls test using a computer program (SigmaStat, San Rafael, CA).

Results

Changes in the Density of 5-HT transporters Following Prenatal Fluoxetine Exposure

Telencephalon. Site-specific alterations in 5-HT transporters were observed in specific telencephalic brain regions following prenatal exposure to fluoxetine (table 1, fig. 1). For example, 5-HT transporters were significanty ($P < .05$; Neuman-Keuls test) increased only in the CA2 (+47%) and CA3 (+38%) areas of the hippocampus in fluoxetine-exposed prepubescent offspring, whereas the density of 5-HT transporters was not altered in either the dentate gyrus or in the CA1 area of the hippocampus (table 1). 5-HT transporters were also significantly elevated in select nuclei of the amygdala in prepubescent offspring prenatally exposed to fluoxetine. For example, 5-HT transporters were significantly increased in the basolateral (+32%; $F_{(1,13)} = 6.05$, $P = .029$) and medial (+44%; $F_{(1,15)} = 6.45$, $P = .023$) amygdaloid nuclei, as determined by two-way ANOVA. However, 5-HT transporters in

TABLE 1

Effect of prenatal fluoxetine exposure on the density of 5-HT transporters in select brain regions within the telencephalon of prepubescent and adult male rat offspring

Data are expressed as fmol of sites/mg of tissue equivalent and represent the mean ± S.E.M. from 3–6 rats per group with each rat within a group being obtained from a different litter. The number of rats per group is shown in parentheses. Prenatal exposure to fluoxetine (10 mg/kg/2 ml s.c. to dams from GD 13-20) significantly increased the density of 5-HT transporters in the CA2 (+47%) and CA3 (+38%) areas of the hippocampus, as well as the basolateral (+32%) and medial (+44%) amygdaloid nuclei in prepubescent progeny. By PD 70, the density of 5-HT transporters was similar between saline- and fluoxetine-exposed offspring across all brain regions examined. 5-HT transporters were labeled with 0.7 nM [^{3}H]citalopram. Nonspecific binding was determined in the presence of 1 μM paroxetine. Quantification of autoradiograms was performed using computerized digital image analysis as described in Materials and Methods. Data were analyzed using a two-way ANOVA followed by a Newman-Keuls *post hoc* test.

Telencephalon	Prepubescent progeny		Adult progeny	
	Saline (N)	Fluoxetine (N)	Saline (N)	Fluoxetine (N)
Cortex				
Cingulate cortex	24 ± 2 (5)	25 ± 3 (5)	32 ± 1[b] (5)	34 ± 3[b] (6)
Entorhinal cortex	24 ± 4 (5)	20 ± 1 (4)	30 ± 1 (5)	30 ± 5 (6)
Frontal cortex	14 ± 2 (5)	14 ± 2 (5)	15 ± 1 (5)	13 ± 1 (6)
Occipital cortex	7 ± 1 (5)	6 ± 1 (4)	6 ± 1 (5)	8 ± 1 (6)
Parietal cortex	13 ± 2 (4)	13 ± 1 (5)	10 ± 1 (5)	9 ± 1 (5)
Retrosplenial granular cortex	12 ± 1 (4)	12 ± 1 (5)	10 ± 1 (4)	14 ± 1 (5)
Temporal cortex	17 ± 4 (5)	14 ± 1 (4)	6 ± 1[b] (5)	8 ± 1 (6)
Hippocampus				
CA1 area of Ammon's horn	9 ± 1 (5)	12 ± 1 (5)	16 ± 2[b] (5)	17 ± 2 (5)
CA2 area of Ammon's horn	19 ± 1 (5)	28 ± 1[a] (4)	29 ± 3 (4)	31 ± 4 (5)
CA3 area of Ammon's horn	21 ± 3 (4)	29 ± 1[a] (5)	37 ± 2[b] (4)	37 ± 2[b] (6)
Dentate gyrus	7 ± 1 (4)	10 ± 1 (5)	10 ± 1 (5)	11 ± 2 (6)
Septum				
Lateral septum, dorsal	20 ± 2 (5)	25 ± 1 (4)	21 ± 3 (4)	25 ± 3 (6)
Lateral septum, intermediate	60 ± 5 (5)	61 ± 3 (4)	57 ± 4 (5)	63 ± 3 (5)
Amygdala				
Basolateral amygdala	71 ± 5 (4)	94 ± 1[a] (4)	112 ± 7[b] (4)	113 ± 5 (5)
Central amygdala	13 ± 2 (5)	15 ± 2 (5)	17 ± 1 (5)	17 ± 2 (6)
Medial amygdala	32 ± 1 (4)	46 ± 3[a] (4)	53 ± 1[b] (5)	50 ± 3 (6)
Basal Ganglia				
Caudate putamen	38 ± 3 (5)	37 ± 2 (5)	33 ± 3 (5)	28 ± 2 (6)
Globus pallidus	86 ± 5 (3)	59 ± 6 (3)	69 ± 2 (3)	56 ± 9 (5)
Ventral pallidum	63 ± 7 (4)	55 ± 4 (3)	70 ± 2 (4)	61 ± 6 (5)

[a] Significantly different from the respective control values in prepubescent progeny ($P < .05$).
[b] Significantly different from the respective values in prepubescent offspring ($P < .05$).

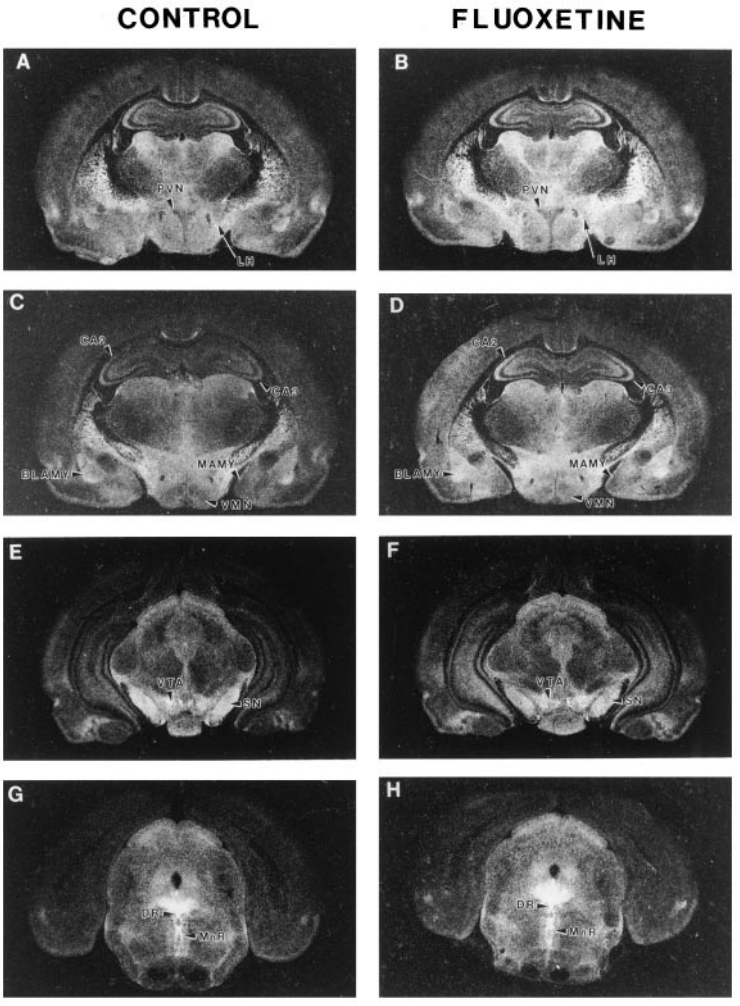

Fig. 1. Dark field autoradiograms demonstrating the effect of prenatal fluoxetine exposure on the density of [^{3}H]citalopram-labeled 5-HT transporters in prepubescent progeny. Representative autoradiograms of control progeny are shown in A, C, E and G. Representative autoradiograms of fluoxetine-exposed progeny are shown in B, D, F and H. White areas indicate brain regions with a high density of 5-HT transporters. Prenatal exposure to fluoxetine resulted in a significant increase in the density of 5-HT transporters in the lateral hypothalamus (LH); the CA2 and CA3 areas of the hippocampus; and in the basolateral (BLAMY) and medial (MAMY) amygdaloid nuclei. In contrast, the density of 5-HT transporters was significantly decreased in the substantia nigra (SN). The density of 5-HT transporters was not altered in either the ventromedial (VMN) or paraventricular (PVN) nuclei of the hypothalamus or in the ventral tegmental area (VTA). In addition, the density of 5-HT transporters in serotonergic cell body regions [dorsal (DR) and median (MnR) raphe] was not altered by prenatal exposure to fluoxetine. 5-HT transporters were labeled with 0.7 nM [^{3}H]citalopram. Nonspecific binding was defined in the presence of 1 μM paroxetine.

the central amygdala, caudate putamen, globus pallidus and ventral pallidum were similar between control and fluoxetine-exposed prepubescent offspring. In contrast, in adult offspring (table 1), prenatal exposure to fluoxetine did not significantly alter the density of 5-HT transporters in any subregion of the hippocampus (CA1, CA2, CA3, dentate) nor in any of the basal ganglia or amygdaloid nuclei examined in the present study (central, basolateral, and medial amygdala; caudate putamen, globus pallidus, and ventral pallidum). In either prepubescent or adult progeny, prenatal exposure to fluoxetine did not alter the density of 5-HT transporters in any of the cortical areas examined (cingulate, frontal, entorhinal, occipital, parietal, retrosplenial granular and temporal cortex). Similarly, the density of 5-HT transporters in lateral septal nuclei (dorsal and intermediate areas) was not affected by prenatal fluoxetine exposure in either prepubescent or adult progeny.

Diencephalon and mesencephalon. As observed in the telencephalon, changes in 5-HT transporters were observed in select regions of diencephalon and mesencephalon only in prepubescent progeny (table 2, fig. 1). However, both increases and decreases in 5-HT transporters were detected. For example, two-way ANOVA indicated a significant elevation in 5-HT transporters in the lateral hypothalamus (+21%; $F_{(1,15)} = 10.14$, $P = .006$) in prepubescent fluoxetine-exposed progeny. In contrast, a significant reduction (−21%; Neuman-Keuls test, $P < .05$) in 5-HT transporter density was detected in the dorsomedial hypothalamic nucleus of prepubescent progeny. However, densities of 5-HT transporters were similar between control and fluoxetine-exposed animals in all other subregions of the hypothalamus examined (anterior, arcuate, paraventricular and ventromedial nuclei; medial mammillary and medial preoptic areas) in prepubescent progeny. In addition to hypothalamic alterations, two-way ANOVA indicated that prenatal exposure to fluoxetine produced a significant decrease in 5-HT transporters in the substantia nigra (−19%; $F_{(1,12)} = 9.76$, $P = .0007$) in prepubescent offspring. Despite the alterations in 5-HT transporters in a number of regions receiving serotonergic innervation, 5-HT transporters, in brain regions composed primarily of serotonin perikarya (*i.e.,* dorsal and median raphe nuclei), were not altered by prenatal exposure to fluoxetine.

In contrast to the changes observed in prepubescent offspring, in adult progeny, there were no changes in 5-HT transporters in the substantia nigra, the ventral tegmenal area, the various subregions of the hypothalamus measured, or in the dorsal and median raphe nuclei following prenatal exposure to fluoxetine (table 2).

Changes in the density of 5-HT transporters as a consequence of maturation. The regional specificity of changes in the density of 5-HT transporters as a consequence of normal maturation (*i.e.,* prepubescent *vs.* adult densities of

TABLE 2

Effect of prenatal fluoxetine exposure on the density of 5-HT transporters in select brain regions within the diencephalon and mesencephalon of prepubescent and adult male rat offspring

Data are expressed as fmol of sites/mg of tissue equivalent and represent the mean ± S.E.M. from 3–6 rats per group with each rat within a group being obtained from a different litter. The number of rats per group is shown in parentheses. Prenatal exposure to fluoxetine (10 mg/kg/2 ml s.c. to dams from GD 13-20) significantly increased the density of 5-HT transporters in the lateral hypothalamus (+21%) in prepubescent progeny. In contrast, the density of 5-HT transporters was significantly decreased in the dorsomedial nucleus of the hypothalamus (−21%) and in the substantia nigra (−19%) in prepubescent progeny. By PD 70, the density of 5-HT transporters was similar between saline and fluoxetine-exposed offspring across all brain regions examined. 5-HT transporters were labelled with 0.7 nM [^{3}H]citalopram. Nonspecific binding was determined in the presence of 1 μM paroxetine. Quantification of autoradiograms was performed using computerized digital image analysis as described in Materials and Methods. Data were analyzed using a two-way ANOVA followed by a Newman-Keuls *post hoc* test.

	Prepubescent progeny		Adult progeny	
	Saline (N)	Fluoxetine (N)	Saline (N)	Fluoxetine (N)
Diencephalon				
Hypothalamus				
Anterior hypothalamus	65 ± 6 (5)	74 ± 4 (5)	54 ± 2 (4)	67 ± 2 (6)
Arcuate nucleus	18 ± 1 (5)	24 ± 1 (4)	28 ± 2^b (4)	22 ± 2 (6)
Dorsomedical nucleus	43 ± 2 (4)	34 ± 1^a (4)	45 ± 3 (5)	44 ± 3† (5)
Lateral hypothalamus	71 ± 3 (4)	86 ± 6^a (5)	62 ± 3 (5)	72 ± 3† (5)
Medial mammillary area	63 ± 5 (5)	62 ± 5 (4)	58 ± 6 (5)	70 ± 7 (6)
Medial preoptic area	35 ± 3 (4)	31 ± 5 (3)	37 ± 4 (5)	34 ± 3 (5)
Paraventricular nucleus	38 ± 3 (5)	43 ± 2 (5)	34 ± 2 (5)	39 ± 3 (6)
Ventromedial nucleus	30 ± 3 (5)	34 ± 1 (5)	35 ± 3 (5)	28 ± 1 (5)
Mesencephalon				
Tegmentum				
Substantia nigra	121 ± 5 (4)	98 ± 3^a (3)	90 ± 5^b (4)	74 ± 8^b (5)
Ventral tegmental area	114 ± 8 (5)	109 ± 6 (4)	79 ± 5^b (5)	86 ± 6^b (6)
Raphe Nuclei				
Dorsal raphe	155 ± 8 (5)	155 ± 14 (4)	138 ± 4 (5)	149 ± 5 (6)
Median raphe	145 ± 10 (5)	134 ± 5 (3)	108 ± 4^b (4)	115 ± 5 (6)

[a] Significantly different from the respective control values in prepubescent progeny ($P < .05$).
[b] Significantly different from the respective values in prepubescent offspring ($P < .05$).

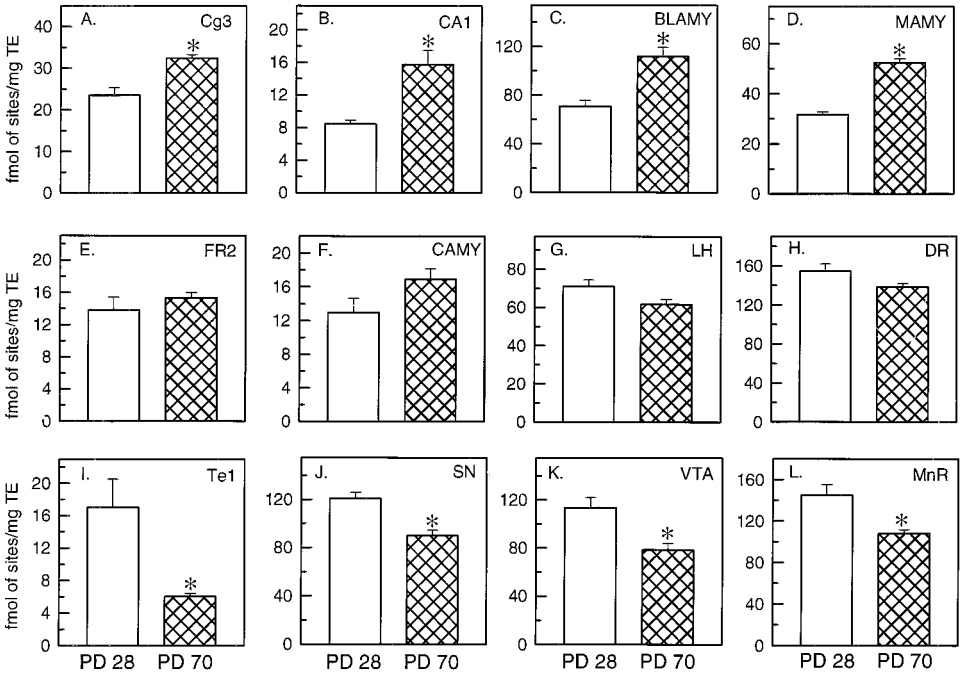

Fig. 2. Representative brain regions in which the density of [^{3}H]citalopram-labeled 5-HT transporters either increased, decreased or remained constant as a consequence of maturation. Data are expressed as fmol of sites/mg tissue equivalent (fmol/mg TE) and represent the mean ± S.E.M. from 4–5 control rats with each rat within a group being obtained from a different litter. Adult (PD 70) control progeny exhibited a significantly greater density of 5-HT transporters in the cingulate cortex (Cg3; *A*; +33%), CA1 (*B*; +78%) area of the hippocampus and in the basolateral (BLAMY; *C*; +58%) and medial (MAMY; *D*; +66%) amygdaloid nuclei than their prepubescent counterparts. The density of 5-HT transporters was similar in prepubescent and adult control progeny in the frontal cortex (FR2; *E*), central amygdala (CAMY; *F*), lateral hypothalamus (LH; *G*) and dorsal raphe (DR; *H*). In contrast, the density of 5-HT transporters was significantly lower in adult progeny in the temporal cortex (Te1; *I*; −65%), substantia nigra (SN; *J*; -26%), ventral tegmental area (VTA; *K*; -31%) and median raphe (MnR; *L*; -25%). 5-HT transporters were labeled with 0.7 nM [^{3}H]citalopram. Nonspecific binding was determined in the presence of 1 μM paroxetine. Data were obtained via the quantification of autoradiograms which was performed using computerized digital image analysis as described in Materials and Methods. Data were analyzed as in tables 1 and 2 using a two-way ANOVA followed by a Newman-Keuls *post-hoc* test. *Significantly different from prepubescent (PD 28) values ($P < 0.05$).

5-HT transporters in control progeny) are shown in figure 2. While, the majority of brain areas examined in the present study exhibited comparable densities of 5-HT transporters at prepubescent and adult ages (tables 1 and 2), notable increases and decreases were observed in several specific neuroanatomic loci. For example, adult control progeny exhibited a significantly greater density of 5-HT transporters than their prepubescent counterparts in the cingulate cortex (+33%; $F_{(1,14)} = 14.56$, $P < .0019$), the arcuate nucleus of the hypothalamus (+ 56%; $P < .05$, Neuman-Keuls test), the basolateral (+58%; $F_{(1,13)} = 37.81$, $P < .0001$) and medial (+66%; $F_{(1,15)} = 26.84$, $P = .0001$) amygdaloid nuclei, as well as in CA1 (+48%; $F_{(1,16)} = 14.68$, $P = .0015$) and CA3 (+76%; $F_{(1,15)} = 27.86$, $P < .0001$) areas of the hippocampus (tables 1 and 2). In contrast, significant age-related reductions were noted in a number of other brain regions (tables 1 and 2; fig. 2). Two-way ANOVA indicated significantly lower densities of 5-HT transporters in control adult offspring within the temporal cortex (−65%; $F_{(1,16)} = 18.91$, $P = .0005$), substantia nigra (−26%; $F_{(1,12)} = 20.12$, $P = .0007$), ventral tegmental area (−31%; $F_{(1,16)} = 19.08$, $P = 0.0005$), and median raphe (−25%; $F_{(1,14)} = 14.56$, $P = .0019$). These changes may represent changes in 5-HT innervation or functional changes in 5-HT neurons in these regions as a consequence of normal maturation.

Discussion

The present study demonstrates that prenatal exposure to fluoxetine produces region-specific alterations in the density of [^{3}H]citalopram-labeled 5-HT transporters in prepubescent offspring. In particular, 5-HT transporter densities were markedly altered in the substantia nigra, as well as in several brain regions which are integral components of the limbic system including subregions of the hippocampus, amygdala, and hypothalamus. The increases and decreases in [^{3}H]citalopram-labeled 5-HT transporters in offspring reflects fluoxetine-induced alterations in either: (1) the extent of serotonergic innervation; (2) the number of transporters present per neuron; or (3) the affinity of the radiolabel for the transporter.

Through the years, many studies have suggested a link between 5-HT innervation density and the amount of specific radiolabeled 5-HT transporters in brain tissue (Battaglia *et al.*, 1987; Battaglia, 1990; D'Amato *et al.*, 1987; Pranzatelli and Martens, 1992). More recently, Descarries *et al.* (1995) reported that quantitative autoradiography of [^{3}H]citalopram-labeled 5-HT transporters in rat brain slices, paralleled treatment induced changes in the density of 5-HT innervation as measured by the number of [^{3}H]5-HT-labeled varicosities. Consistent with previous research, these authors concluded that radiolabeled citalopram could serve as a quantitative marker for 5-HT innervation *in vitro*. Thus, it is likely that alterations in [^{3}H]citalopram-labeled 5-HT transporters in prepubescent offspring reflect drug-induced changes in the extent of serotonergic innervation of the affected brain regions. However, data in transfected cell lines also suggests that the number of 5-HT transporters present at the cell surface can be regulated by the stimulation of protein kinases in a similar way to what has more traditionally been described for the 5-HT receptors (Qian *et al.*, 1997). Hence, it is also possible that the reductions in [^{3}H]citalopram-labeled transporters produced by prenatal fluoxetine may reflect changes in the number of transporters present per neuron within a brain region, rather than alterations in the extent of serotonergic innervation. Alterations in the number of 5-HT transporter sites per neuron could result from changes in the stability of the protein or intracellular trafficking and insertion into the plasma membrane. Finally, the present study utilized a single concentration of radioligand below the K_D value for the radiolabel to assess 5-HT transporter binding. Because this approach renders radioligand binding sensitive to changes in either the affinity and/or density of 5-HT transporters, we cannot rule out the possibility that prenatal fluoxetine exposure altered the affinity of the transport protein for the radioligand in 28-day-old offspring. However, this possibility is unlikely, as one would expect that changes in the affinity of the transporter would result in unidirectional changes (either all increases or all decreases) in citalopram-labeling of 5-HT transporters which would persist into adulthood. However, we report both increases and decreases in 5-HT transporters in specific nuclei which are present at prepubescent but not adult ages. Consistent with this contention, Montero *et al.* (1990) identified reductions in [^{3}H]imipramine-labeled 5-HT transporters following prenatal fluoxetine exposure in the absence of alterations in the affinity of the ligand for the transport protein.

As previously discussed, the underlying mechanism for the reductions in the density of [^{3}H]citalopram-labeled 5-HT transporters cannot be definitively concluded from the present study. However, regardless of the mechanism responsible for the differences in 5-HT transporter density (*i.e.* alterations in the extent of serotonergic innervation, or in the number of 5-HT transporters per nerve terminal), one can speculate that fluoxetine-induced changes in 5-HT transporter numbers may result in alterations in serotonergic neurotransmission within the brain regions affected by the prenatal treatment. This possibility is supported by the fact that the 5-HT transporter plays a key role in regulating extracellular concentrations of 5-HT and consequent receptor activation (Schroeter and Blakely, 1996).

The prenatal fluoxetine-induced alterations in [^{3}H]citalopram-labeled 5-HT transporters appears to be age-dependent, since no significant differences in the density of 5-HT transporters were observed in adult offspring. In this regard, the present studies are consistent with the age-dependent changes in the density of [^{3}H]imipramine-labeled 5-HT transporters reported by Montero *et al.* (1990) following prenatal exposure to fluoxetine (2.5 mg/kg/day). However, the present data appear to contrast with our previous report indicating that prenatal fluoxetine exposure did not alter the density of 5-HT transporters in homogenates of various forebrain regions (Cabrera and Battaglia, 1994; Cabrera-Vera *et al.*, 1997). Taken together, these studies indicate that prenatal fluoxetine exposure produces subtle region-specific alterations in select brain 5-HT pathways that can not be readily discerned from homogenate assays, which are more likely to detect global, rather than discrete, changes in neurotransmitter systems within specific brain regions.

While differences in tritium quenching between prepubescent and adult progeny (due to age-dependent changes in lipid composition of the brain) could be postulated to produce artifactual differences between prepubescent and adult off-

spring 5-HT transporter densities, this is unlikely to have contributed to the autoradiographic differences reported herein. This contention is supported by several observations: (1) autoradiographic analysis of adjacent sections with other tritiated radioligands does not reveal the presence of a consistent pattern of quenching across the two age groups in any specific brain region (Cabrera *et al.*, 1995); (2) the majority of the radioactive signal obtained in the regions analyzed in the present study results from localization of the transporter in 5-HT neuronal cell bodies or terminals rather than from axons of passage; (3) both age-related increases and decreases in [^{3}H]citalopram-labeled transporters were observed, whereas developmental delays in myelination would be expected to alter the signal in the same direction across all brain regions. In addition, developmental differences in the pattern of ^{3}H-citalopram-labeled 5-HT transporters in prepubescent rats has previously been reported (D'Amato *et al.*, 1987).

The absence of changes in 5-HT transporters in adult animals prenatally exposed to fluoxetine observed in the present study may initially suggest that 5-HT systems have "normalized" by adulthood. However, functional deficits in 5-HT nerve terminals may be present in adult animals in the absence of alterations in the density of 5-HT transporters. Consistent with this hypothesis, Battaglia (1990) demonstrated that, in cortex of rats recovering from MDMA-induced lesion of serotonergic axons, following an initial 90% reduction in 5-HT transporters, 5-HT transporters reached control levels after 1 year. However, 5-HT levels remained markedly below control values suggesting a functional alteration in presynaptic 5-HT neurons. Furthermore, we recently reported that midbrain 5-HT content was significantly reduced in adult progeny prenatally exposed to fluoxetine (Cabrera-Vera *et al.*, 1997). This reduction in 5-HT content occurred in the absence of concomitant changes in the density of dorsal and median raphe 5-HT transporters as reported in the present studies. We have also previously reported a significant attenuation of the ability of the 5-HT releasing drug PCA to reduce 5-HT content in midbrain of adult offspring prenatally exposed to fluoxetine; providing further evidence of a functional impairment in 5-HT neurons in this region (Cabrera-Vera *et al.*, 1997). Taken together, these data suggest that whereas there may be a recovery from the changes in 5-HT transporter densities in prepubescent rats following maturation, the functional status of 5-HT terminals in brain regions affected by prenatal fluoxetine exposure may remain compromised in adult progeny.

Consistent with the hypothesis of functional changes in 5-HT pathways in adult progeny, we previously reported that prenatal fluoxetine exposure reduced hypothalamic 5-HT$_{2A/2C}$ receptors and the 5-HT$_{2A/2C}$ receptor-mediated adrenocorticotropin response selectively in adult, but not prepubescent progeny (Cabrera and Battaglia, 1994). Hence, our previous data suggested a delayed onset of perturbations in postsynaptic brain 5-HT pathways following prenatal fluoxetine exposure. According to the classic theory of receptor regulation, receptor number and/or function is altered secondary to changes in a presynaptic stimulus. Thus, fluoxetine-induced changes in postsynaptic 5-HT receptor systems may be due, in part, to alterations in the extent of serotonergic innervation or to the altered functional status of 5-HT neurons which occur early in the life of the offspring.

In summary, the present studies provide additional evidence that prenatal exposure to the selective 5-HT uptake inhibitor fluoxetine (Prozac) results in biochemical alterations in brain 5-HT pathways in offspring. The biochemical alterations reported in the present study (*i.e.,* increases and decreases in the density of 5-HT transporters) are region-specific. As the current data suggest that limbic regions are particularly vulnerable to prenatal fluoxetine exposure, further study of serotonergic function in limbic brain regions might prove to be a particularly fruitful avenue of research. In addition, as activation of the limbic system mediates emotional responses, "fight or flight" reactions, as well as food finding and sexual behaviors, limbic-based behavioral examinations may also be warranted. As 5-HT transporters play a key role in regulating extracellular concentrations of 5-HT and thereby influence the duration and extent of 5-HT receptor activation following neurotransmitter release, the present data suggest the potential for functional alterations in serotonergic neurotransmission within select brain regions in young male offspring exposed *in utero* to fluoxetine. The nature of the functional consequences of these alterations in 5-HT systems, and the implication for drugs which target 5-HT transporters for their therapeutic efficacy, remain to be elucidated. In addition, it remains to be determined whether the observed changes in brain 5-HT pathways, will be generalizable to other SSRIs, and whether human offspring prenatally exposed to fluoxetine might exhibit similar changes in brain 5-HT pathways. Because alterations in brain 5-HT pathways have been implicated in the etiology of a variety of clinical disorders (*e.g.,* depression, aggression, anxiety), one could speculate that should human offspring exhibit neurochemical alterations similar to those described herein, these individuals may be particularly susceptible to developing psychiatric disorders involving dysfunctional 5-HT pathways. Alternatively, prenatal fluoxetine-induced changes in 5-HT systems in human offspring could alter their responsiveness to therapeutic interventions which modulate brain 5-HT systems. While these possibilities are intriguing they will require a substantial amount of additional research to determine their validity.

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