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Research Report

Selective serotonin reuptake inhibitor disrupts organization of thalamocortical somatosensory barrels during development

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Abstract

To further investigate the role of the transiently expressed serotonin (5-HT) transporter (5-HTT) in the development of thalamic fibers projecting to cortical barrels and the potential developmental changes in neuronal circuitry caused by a selective serotonin reuptake inhibitor (SSRI), paroxetine (5 mg/kg, twice daily, s.c.) or saline was administered to rat pups from postnatal day 0 (P0) to P8. Pups were perfused on P8 for 5-HT immunostaining (-im) to confirm the 5-HT uptake blockade, and 5-HTT-im and phospholipase C-β1 (PLC-β1)-im to label the thalamic afferents to barrels and barrel cells respectively. Paroxetine treatment completely blocked 5-HT uptake into the thalamocortical fibers as indicated by the negative 5-HT-im in cortical barrel areas. Organization of thalamic afferents to barrels, indicated by 5-HTT-im or PLC-β1, was altered in paroxetine-treated pups in the following manners: (1) segregation of thalamocortical fibers was partially disrupted and thalamocortical fibers corresponding to anterior snouts and row A mystacial vibrissae were fused; (2) sizes of the unfused thalamocortical fiber patches related to the long caudal vibrissae in rows B, C, D and E were significantly decreased without changes in the brain weights and cortical areas representing these vibrissae; and (3) thalamocortical fibers corresponding to C4 and D4 vibrissae tended to be closer to each other along the arc while the relative positions of thalamocortical fibers related to the rest of the vibrissae were normal. Our study demonstrated that 5-HTT plays an important role in the refinement, but not the formation, of barrel-like clusters of thalamocortical fibers and that the development of neural circuitry in rodent somatosensory cortex was affected by exposure to a SSRI during thalamocortical synaptic formation.

Theme: Development and regeneration
Topic: Sensory system

Keywords: Serotonin; Serotonin transporter; Thalamocortical fiber; Barrel; Paroxetine

1. Introduction

The barrel field in the rodent somatosensory cortex has been used as an excellent model to study the roles of various intrinsic and extrinsic factors on the development and plasticity of the neocortex because of its topographic whisker representation (for review see Ref. [19,21]). The barrels receive thalamic afferents mainly from the ventrobasal complex (VB) [9,18,45]. These VB thalamocortical fibers arborize corresponding to each barrel and form the barrel-like clusters [9,17,18,45] prior to the occurrence of cytoarcitecturally definable barrels during the first postnatal week, and these fibers play a role in inducing the formation and differentiation of barrels [12,16,41].

Thalamocortical fibers from VB projecting to cortical barrels transiently uptake and store Serotonin (5-HT) but do not synthesize it during the first two postnatal weeks, as shown by the transient expression of functional serotonin transporters (5-HTT) in the axons and terminals of these fibers and vesicular monoamine transporters 2 ($VMAT_2$) in thalamic neurons [7,24,25,47]. The peak of 5-HTT expression in these fibers is at P5–P8 [29,47]. $5\text{-}HT_{1B}$ receptors are also transiently expressed in the VB thalamocortical fibers following a similar time course to those of 5-HTT [2,26,29].

Pharmacological or genetic manipulation of 5-HT levels and these 5-HT molecules have been demonstrated to

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affect the development of barrel-like clusters of thalamocortical fibers as well as cortical barrels. An excess of extracellular 5-HT disrupts the barrel-like distribution of thalamocortical fibers and barrel formation as shown in both monoamine oxidase A (MAO-A) inhibitor-treated pups [42] and MAO-A knock-out (KO) mice [8]. 5-HT depletion with 5,7-dihydroxytryptamine decreases the cross-sectional areas of patches of thalamocortical fibers to barrel cortex [3,36]. Pharmacological activation of 5-HT_{1B} receptors has been shown to desegregate the thalamocortical fibers to barrel cortex [46]. The barrel pattern formation is greatly disrupted in 5-HTT KO mice [32]. However, the role of the transiently expressed 5-HTT on the development and plasticity of thalamocortical fibers projecting to barrels during the critical postnatal period needs to be further investigated.

In this study, paroxetine, a selective serotonin reuptake inhibitor (SSRI), was used to block the uptake of 5-HT into thalamocortical fibers during the first postnatal week and the effects of the 5-HT uptake blockage on the organization of thalamic afferents to cortical barrels were assessed. SSRIs have been widely used for the treatment of some psychiatric disorders such as depression and obsessive-compulsive disorder; recent review see Ref. [6]. The influence of SSRI treatment on the development of neural circuitry in neocortex has been little studied. Therefore, it is our interest to investigate the potential changes of neural circuitry in the neocortex induced by SSRI administration during this developmental stage (first postnatal week in rat pups), which may correspond to the third trimester of human pregnancy.

2. Material and methods

2.1. Animals

Experiments were conducted on newborn Sprague–Dawley (SD) rat pups (Harlan, Indianapolis, IN). Timed-pregnant SD rats during mid-gestation were purchased from Harlan and single-housed at the Indiana University Laboratory Animal Research Center (LARC) vivarium with a controlled climate (temperature 22 °C, 30% humidity) and 12:12 normal light–dark cycle. The day of birth of pups was counted as postnatal day 0 (P0).

2.2. Treatments

Paroxetine (5 mg/kg, Smith Kline Beecham Pharmaceuticals) or saline was administered subcutaneously into the backs of rat pups using 30-gauge needles. Injections were carried out twice a day from at least 8 h after birth on P0–P8. The total of 18 pups came from three different litters (litter sizes varied from 10 to 12 pups) and six pups from each litter were randomly divided into an experimental and a control group.

2.3. Tissue sectioning

Pups were anaesthetized with ketamine (50 mg/kg, i.p.) on P8 and perfused transcardially with 0.9% sodium chloride followed by fresh 4% paraformaldehyde in phosphate buffer. Immediately after perfusion, brains were weighed. Cortices were dissected, flattened by 2 mm spacers and postfixed in 4% paraformaldehyde at 4 °C. Tissue for 5-HT immunocytochemistry (-im) were processed within 3 days after fixation. Tangential 50 μm sections were cut using a vibrating microtome (Leica) and the first 15 sections were collected sequentially.

2.4. Immunocytochemistry

Three immunocytochemically stained markers were used in this study, 5-HT, 5-HTT, and phospholipase C-β1 (PLC-β1). The 5-HTT is expressed in the entire thalamocortical fibers and their terminals outline the somatosensory barrels including the whisker barrels [47]. The 5-HT staining represents the 5-HT being up-taken into the thalamocortical fibers; in the treatment with paroxetine, its reduction or absence confirms the effect of the SSRI in blocking 5-HT uptake. For a further verification of barrel filed, a subset of tissues was stained with PLC-β1. PLC-β1 is an intracellular signaling molecule, which has been relate to development plasticity in the cat visual cortex, and is concentrated in an intermediate compartment-like organelle, the botrysome [20]. The PLC-β1 expression pattern is shown to be specific to the post-synaptic developing barrel field and is most distinct at postnatal days 4–9, but not at adult stage [15].

Free-floating sections from one hemisphere were stained sequentially for 5-HT, and sections from the other hemisphere of the same animal were stained for 5-HTT. No noticeable difference in lateralization has been found between hemispheres. Sections were washed in PBS and incubated in PBS containing 1.5% normal sheep serum (NSS) and 0.3% Triton X-100 (TX) for 30 min prior to applying antibodies. The sections were incubated with either rabbit anti-5-HTT (1:750, [48]) rabbit anti-5-HT (1:250, Incstar) for 36 h at 4 °C. Then, sections were rinsed three times in PBS and incubated in sheep anti-rabbit IgG (1:100, The Biotech Source) for 1.5 h and washed three times in PBS. The third antibody was rabbit peroxidase anti peroxidase (PAP) (1:500, Jackson Immunoresearch Lab). 5-HT or 5-HTT-im was visualized in a reaction with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) and 0.003% H_2O_2.

For PLC-β1 staining, sections were blocked for 30 min with 3% normal sheep serum (NSS), 0.3% (v/v) Triton X-100, in PBS and then incubated at 4 °C for 2 days in primary antibody rabbit anti-PLC-β1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted (1:250) in 1.5% NSS, 0.3% TX in PBS. After three washes in PBS, the Sternberger's peroxidase-anti-peroxidase (PAP) indirect enzyme-method was used for detection of PLC-β1-im.

The sections were then incubated sequentially in sheep-anti-rabbit and rabbit-PAP antibodies for 1 h each. Between incubation with antibodies, tissues were washed three times with PBS each with 10 min. The peroxidase activity was revealed with 0.05% (w/v) DAB (Sigma) and 0.6% (w/v) $Ni(NH_4)(SO_4)$ in 0.05 M Tris–HCl buffer (pH 7.6) supplemented with 0.003% H_2O_2.

2.5. Morphometric measurements and image analysis

Two barrel subfields, cortical posteromedial barrel subfield (PMBSF) and anteriolateral barrel subfield (ALBSF) were observed in this study. All the following measurements refer to PMBSF, which correspond to the long caudal vibrissae. The 5-HTT-im labeled sections were digitally scanned with a Spot II camera into NIH Image software. Only the sections within layer IV (usually four sections with clear barrel visualization) of each animal were used for image analysis and measurements. Brightness and contrast were adjusted in each section to achieve the sharpest boundaries possible between the thalamocortical fiber patches with intensive 5-HTT-im staining and the septa regions with negative 5-HTT-im staining.

2.5.1. The overall area of cortical posteromedial barrel subfield (PMBSF)

The external contour of the cortical area of PMBSF was outlined by connecting the outermost points of every barrel in PMBSF smoothly using a curved line as shown in Fig. 1. The area encircled was measured in every section as the overall cortical area of PMBSF (the area including all barrel patches and inter-barrel areas in PMBSF) and the averaged value in these sections of each animal was used for statistical analysis.

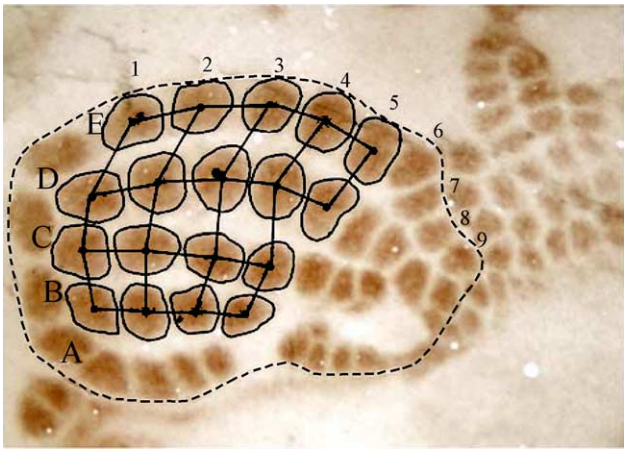

Fig. 1. A representative 5-HTT-im section in cortical PMBSF of a normal P8 rat. Letters A, B, C, D and E represent the five rows and numbers from 1 to 9 represent the nine arcs in PMBSF. Cortical areas of overall PMBSF are outlined with dotted line; 5-HTT-im patches corresponding to B1–B4, C1–C4, D1–D5 and E1–E5 barrels are encircled with solid lines. The geometrical X–Y center of each circled barrels was determined by the NIH Imaging software as shown by the dotted outline. The shortest distance between adjacent centers of barrels is defined as line segment; the sum of the lengths of the line segments is referred to as total line length.

2.5.2. The sizes of thalamocortical fiber patches

The barrels in PMBSF are identified individually by row (A–E) and column (or arc, 1–9) as shown in Fig. 1. Only areas of thalamocortical fiber patches related to B1–B4, C1–C4, D1–D5 and E1–E5 vibrissae were measured because these patches were relatively large and had sharp boundaries, while patches related to barrels in row A were frequently fused in paroxetine treated pups. Cross-sectional areas of patches of 5-HTT-im labeled thalamocortical fibers corresponding to B1–B4, C1–C4, D1–D5 and E1–E5 barrels were circled along their boundaries, the edges of which were defined as areas with at least 90% of the 5-HTT-im intensities in the barrel centers (Fig. 1).

The areas of circled patches were measured using NIH Image software. To compare the sizes of patches that contain the most abundant thalamocortical fibers among the serial sections, the largest measurement for individual thalamocortical patches of each animal was used for analysis, the same method reported by the Bennett-Clarke group [3]. The sum of the largest measurements for all the circled barrel patches in each animal was also calculated to estimate the extent of changes in size when cortical barrel patches corresponding to long caudal vibrissae were considered as a whole.

2.5.3. The distance between adjacent thalamocortical fiber patches and total length among patches

The distance between adjacent patches was defined by the length between the center of patch pairs. The X–Y center of each circled patch was determined using NIH Image software (Fig. 1). The "line segment" refers to distance between the centers of adjacent barrels within the same row (such as between the centers of B1 and B2, B3 and B2) as well as within the same arc (between the centers of B1 and C1, D1 and C1) (Fig. 1). The length of each line segment was measured in every section and averaged in each animal to contribute a single value for analysis. The line segment provides an estimation of the relative position of these thalamocortical fiber patches.

The sum of the lengths of all line segments within the barrel field (e.g. PMBSF) was also calculated and referred to as 'total line length' (Fig. 1). Total cortical area representing the long caudal vibrissae can be measured directly by outlining the entire external contour of these thalamocortical fiber patches. However, the direct measurement of total cortical area could be affected by changes in both the size of the outermost barrel patches and the cortical areas devoted to these thalamocortical fibers. Therefore, the total line length was used for relative estimation of the cortical areas corresponding to these thalamocortical fibers after controlling the differences in size of outermost patches among different groups [3,31,37,38].

2.6. Statistical analyses

Results of the quantitative analysis were compared between paroxetine-treated and control groups using Student's *t*-test. To investigate whether the size of these thalamocortical fiber patches in the four different rows were affected to different extents by paroxetine treatment, the percentages of reduction in the areas of thalamocortical fiber patches were compared among the four rows in the paroxetine-treated group using one-way analysis of variance (ANOVA). $P < 0.05$ was accepted as the level of statistical significance.

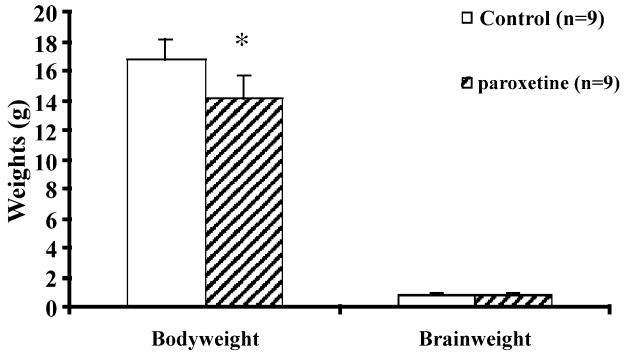

Fig. 3. Brain weight was not altered in paroxetine-treated pups compared to those in normal controls ($P > 0.05$), although body weight was significantly reduced. *$P < 0.05$. Error bars = Standard Deviation.

3. Results

3.1. 5-HT immunostaining in thalamocortical fibers

Two sets of 5-HT-im fibers were found in the somatosensory cortical areas: coarse 5-HT-im fibers (>1.5 μm in thick segment) with varicosities sparsely distributed around cortices, and fine 5-HT-im fibers (< 1 μm, all fibers in the barrel area) without obvious varicosities distributed in strict correspondence with barrels. The coarse fibers were traced from 5-HT neurons, the fine ones from thalamic neurons [47]. The fine whisker-specific patches of thalamocortical fibers were labeled positively with 5-HT-im in the control group (Fig. 2A), but negatively in the paroxetine-treated group (Fig. 2B). In both groups of pups, the coarse 5-HT fibers originating from raphe nuclei were still present (Fig. 2D), as also shown in the control group (Fig. 2C). Therefore, paroxetine treatment (5 mg/kg/injection, twice daily, s.c.) from P0–P8 completely blocked the uptake of 5-HT into thalamocortical fibers resulting in a complete loss of the fine fiber vibrissae-related pattern. Paroxetine treatment caused a reduction in 5-HTT-im at 6 h after administration [4]. It is worthy of mention that short-term (hours) treatment only caused reduction of fine fiber 5-HT immunoreactivity and the vibrissae-related pattern remained visible in thicker fibers [4], while longer term paroxetine treatment (days) completely eliminated the 5-HT-im vibrissae-related pattern.

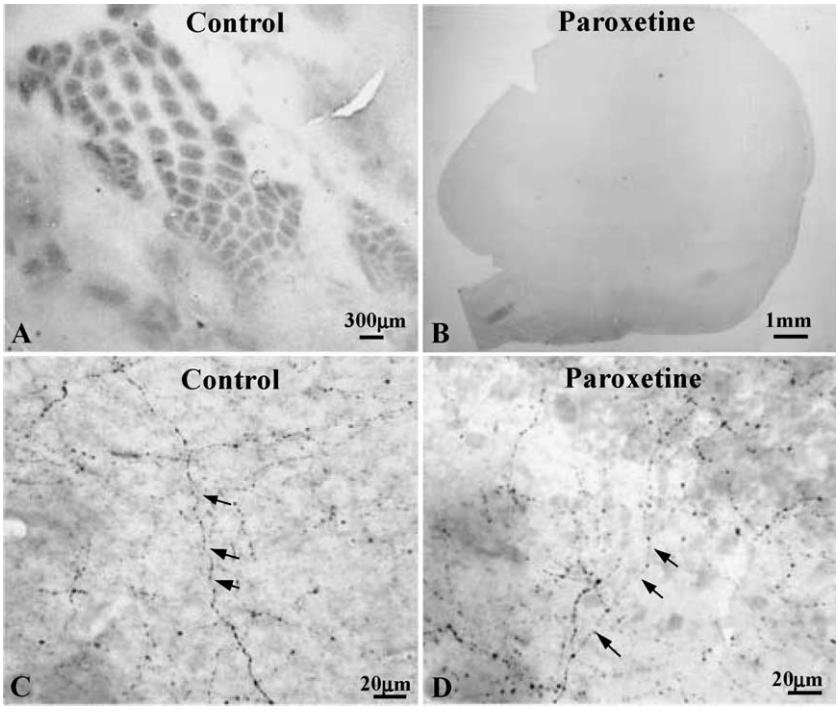

Fig. 2. The serotonin-im (5-HT-im) fibers at layer IV of cortical barrel. The characteristic patchy pattern of extremely fine 5-HT-im thalamocortical fibers with small or no varicosities corresponding to future barrels is found in control (A), but disappeared in paroxetine-treated animals (B). However in higher magnification, the thick 5-HT-im fibers with large varicosities (arrows) were present in both control (C) and paroxetine-treated animals (D). These thick 5-HT-im fibers often found outside the patches are known to be derived from 5-HT neurons in the ascending raphe. Scale bars: A = 300 μm; B = 1 mm; C, D = 20 μm.

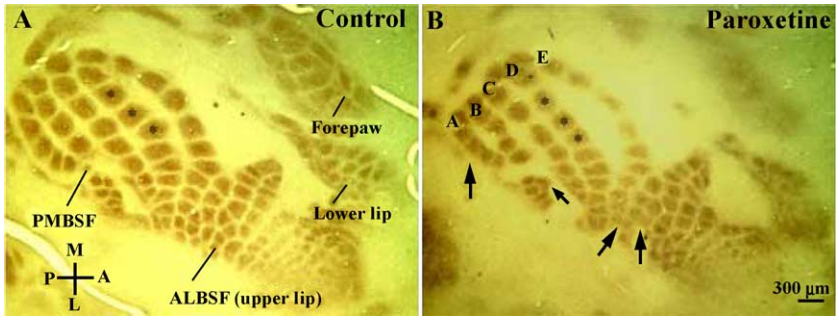

Fig. 4. Representative low magnification photographs show 5-HTT-im thalamocortical fiber patches in somatosensory barrel cortex of both control (A) and paroxetine-treated animals (B). The presence of barrel-like clusters of 5-HTT-im thalamocortical fibers in the paroxetine-treated animal allow for analysis of thalamocortical barrel morphometry. The area of 5-HTT-im thalamocortical fiber patches is smaller in the paroxetine-treated than the control animals (e.g. corresponding stars between A and B). On the contrary, although smaller in size, the segregation of thalamocortical fiber patches was blurred among some patches in the paroxetine-treated animal (B, arrows; detail see next figure). Scale bars: A, B = 300 μm.

3.2. Brain weights and body weights

There was no significant difference in brain weight between paroxetine treated (0.84 ± 1.08 g, $n = 9$) and control groups on P8 (0.83 ± 1.05 g, $n = 9$) ($P > 0.05$) (Fig. 3). The body weights of paroxetine treated pups on P8 were significantly reduced by 14.8% compared to those of normal controls ($P < 0.05$, Fig. 3).

3.3. De-segregation of thalamocortical fibers projecting to the cortical barrels

Normal segregation of thalamocortical fiber patches (labeled by 5-HTT-im) was significantly altered in selective patches of PMBSF in the paroxetine-treated pups (Fig. 4, arrows). Adjacent patches of thalamocortical fibers related to barrels in row A overlapped in paroxetine-treated pups (Fig. 5). Patches of thalamocortical fibers in ALBSF corresponding to anterior snout (AS) were also frequently fused (Fig. 5).

3.4. Overall area and total line length of cortical PMBSF

Overall area of cortical PMBSF (including areas of all barrel patches and interbarrel cortex) was reduced by 11.5% in paroxetine-treated pups as compared with controls ($P < 0.05$). The mean area of paroxetine-treated pups was 3.62 ± 0.34 mm^2 ($n = 9$) and that of controls was 4.09 ± 0.26 mm^2 ($n = 9$) (Fig. 6). The areas of cortex representing B1–B4, C1–C4, D1–D5 and E1–E5 vibrissae were not

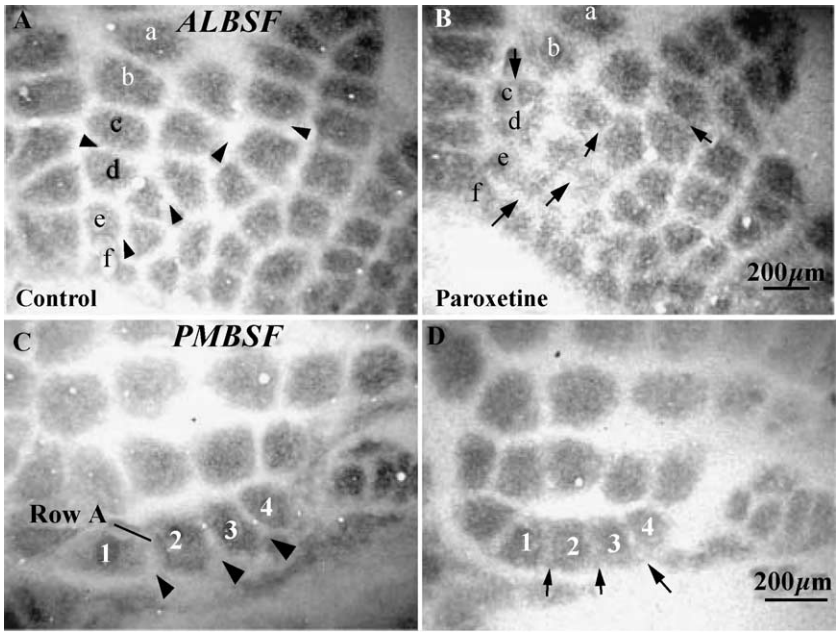

Fig. 5. Desegregation of thalamocortical fiber patches in corresponding barrels. Barrel pattern of 5-HTT-im thalamocortical fibers corresponding in ALBSF of control (A) and paroxetine-treated animals (B), and in PMBSF of control (C) and paroxetine-treated animals. De-segregation was found in some adjacent thalamocortical fiber patches in paroxetine-treated animals (B, D, arrows) as compared to their segregated counterparts in control (A, C, arrowheads). An example is shown in barrels row A (C, D). The space between barrows (septum) is narrower in deseggregated pairs. Scale bars: A, B, C, D = 100 μm.

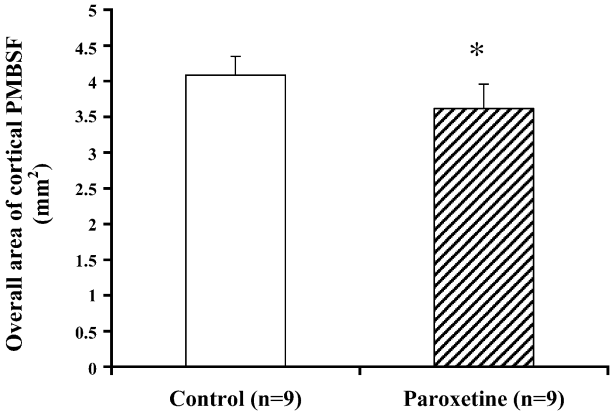

Fig. 6. The overall size of cortical PMBSF was significantly decreased in paroxetine-treated pups compared to that of normal controls. *$P<0.05$. Error bars = Standard Deviation.

altered in the paroxetine-treated group ($P>0.05$) as estimated by the total line length. The total line length of the paroxetine-treated group and the control group was 10.04 ± 0.45 mm ($n=9$) and 10.4 ± 0.66 mm ($n=9$), respectively ($P>0.05$).

3.5. Cross-sectional areas of thalamocortical fiber in PMBSF

The sum of the areas of the measured patches in PMBSF was decreased by 22.5% in paroxetine-treated pups (1.1 ± 0.13 mm^2, $n=9$) compared to those of normal controls (1.42 ± 0.12 mm^2, $n=9$) ($P<0.05$, Fig. 7B). The cross-sectional areas of individual patches of thalamocortical fibers corresponding to B1–B3, C1–C3, D1–D4 and E1–E5 barrels in PMBSF were significantly smaller than

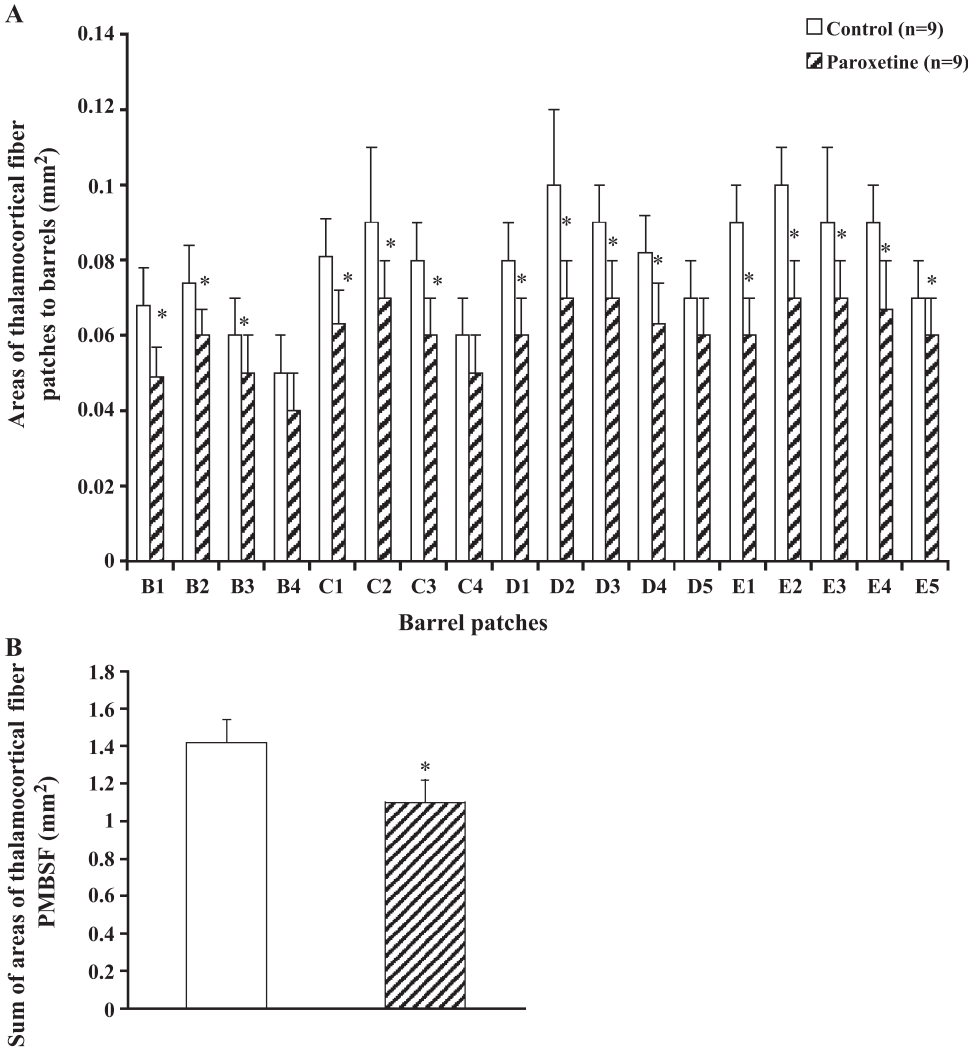

Fig. 7. (A) The cross-sectional area was significantly reduced for each patch of thalamocortical fibers corresponding to B1–B3, C1–C3, D1–D4 and E1–E5 barrels ($P<0.05$), but not to B4, C4 and D5 ($P>0.05$) in paroxetine-treated pups. *$P<0.05$. (B) The sum of cross-sectional areas of thalamocortical fiber patches corresponding to B1–B4, C1–C4, D1–D5 and E1–E5 barrels was significantly smaller in paroxetine-treated pups than that of normal controls. *$P<0.05$. Error bars = Standard Deviation.

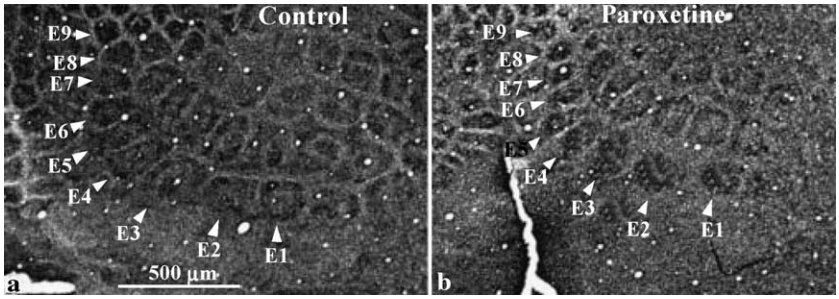

Fig. 8. PLC-β1-im barrels in normal control (a) and paroxetine-treated animals. The sizes of PLC-β1-im patches were decreased in paroxetine-treated (b) as compared to those of normal control animals (a) in rows E2–E9 of PMBSF barrels (arrowheads). Scale bars: a, b = 500 μm.

those of normal controls ($P < 0.05$, Fig. 7A). The extent of reduction in these thalamocortical fiber patches ranged from 13.8% (E1) to 28.8% (D2). The percentages of reduction in cross-sectional area of these patches in each row were similar when compared among these four rows (the mean percentages of reductions for row B, C, D and E were 19.7%, 24.2%, 24.4% and 24.8%, respectively, $P > 0.05$). The areas of thalamocortical fiber patches corresponding to the relatively rostral vibrissae such as B4, C4, D5 in paroxetine-treated groups tended to be smaller than those in control group, but did not reach a statistically significant level ($P > 0.05$, Fig. 7A).

Similar result was found in the PLC-β1-im barrels. The barrels in the most prominent PLC-β1-im row E were compared between the control and paroxetine-treated animals. The size of PLC-β1-im patches decreased in paroxetine-treated as compared to those of normal control animals (Figs. 8 and 9).

3.6. The distance between thalamocortical fiber patches

Within the same row, the distances between the centers of two adjacent thalamocortical patches between arcs (columns) (corresponding to B1-B4, C1-C4, D1-5 and E1-5) were not significantly different between paroxetine-treated and control groups (such as between B1 and B2 barrels, $P > 0.05$). Within the same arc, the distances between the centers of two adjacent row patches (such as B1 and C1) in paroxetine-treated did not differ from those of control groups for most of the patches except for C4 and D4. The distances between the centers of C4 and D4 in the paroxetine-treated group were decreased by 15.8% (0.373 ± 0.003 mm, $n = 9$) compared to those in the control group (0.443 ± 0.004 mm, $n = 9$) ($P < 0.05$).

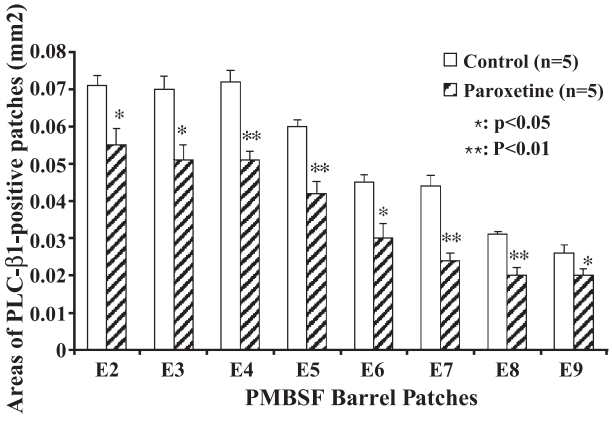

Fig. 9. Histograms showed the measurement of cross-sectional areas of PLC-β1-positive barrels in row E2–E9 of PMBSF. The areas of PLC-β1-im patches corresponding to E2–E9 barrels were significantly decreased in paroxetine-treated pups as compared to those of normal controls (**$p < 0.01$ and *$p < 0.05$). Error bars = SEM.

4. Discussion

Results of this study demonstrated that paroxetine exposure to rat pups during the first postnatal week did not change the brain weight and cortical area representing the long caudal vibrissae, but did affect the organization of thalamic projections to the cortical barrels in the following manners. (1) The patches of thalamocortical fibers projecting to the barrels were formed but the segregation was regionally disrupted. Overlapping of thalamocortical fiber patches were frequently found in adjacent barrels in anterolateral barrel subfield (ALBSF) and in row A of PMBSF. (2) The size of overall PMBSF (Fig. 6) and size of the segregated thalamocortical fiber patches corresponding to the long caudal vibrissae in row B, C, D and E were significantly decreased (Fig. 7). (3) Distance between the centers of thalamocortical fiber patches was largely unchanged, except barrels corresponding to the C4 and D4 vibrissae tended to be shorter in addition to their reduced patch sizes.

4.1. Methodological considerations

The advantage of using 5-HTT-im as marker is that it reveals thalamocortical fibers and delineating their patches in the cortical barrels independent of the variable, the 5-HT, in the current paradigm. One potential limitation in using 5-HTT-im to visualize thalamocortical fibers for morphometric analysis in this study is that chronic treatment of the SSRI fluoxetine to cultured embryonic thalamic

neurons decreases their total 5-HTT protein level [44]. However, the effects of SSRIs on altering 5-HTT in vivo have not been reported, and the level of mRNA for 5-HTT in the raphe nucleus was not altered by chronic paroxetine treatment [1]. We measured the mean density of 5-HTT-im in thalamocortical fibers related to D4 barrel in both paroxetine-treated and control group and did not find a significant difference between these two groups (data not shown). Furthermore, extension of 5-HTT-im fibers beyond patches in the desegregated PMBSF and ALBSF in the paroxetine treated group does not support decreased 5-HTT labeling on thalamocortical fibers. Furthermore, we verified the barrel size with another marker PLCβ1 which labels the post-synpatic neurons. The result of PLCβ1 study is in agreement with those corresponding barrels revealed by 5-HTT-imunostaining. Other makers for thalamocortical fibers, such as DiI or AChE, would provide further confirmation, however DiI is not reliable for quantitation due to its variable infiltration rate. AChE is a workable marker, but we did not adopt AChE because of the wide differential expression of this marker in primary somatosensory cortex of perinatal rats, mice, and hamsters [5]). It would not be suitable for comparison between rat and mouse in our future studies.

To investigate the extent of arborization of thalamocortical fibers, we used the largest measurement of cross-sectional area for an individual thalamocortical fiber patch of each animal for statistical analysis, the same method reported by the Bennett-Clarke group [3]. Another option is to measure the size of barrel-like clusters of thalamocortical fibers using the Stereological Method, which applies mathematical methods to relate the two-dimensional (2-D) measurement obtained on section containing a structure to the three-dimensional (3-D) size of the structure (for review see Ref. [39]), or volume. Systematic random sampling of serial sections and then point counting for each section could be used for unbiased sampling.

4.2. Paroxetine treatment vs. 5-HTT /MAO-a knockout

5-HTT knockout (KO) or MAO-A KO mice demonstrate a very different disruption of the barrel pattern as compared to the rat pups treated with paroxetine. The barrel pattern is almost completely absent except some barely visible caudal barrels in 5-HTT- [32] or MAO-A KO mice [35]; on P7 most PMBSF barrels fuse together to form a sort of undifferentiated "ribbon" within layer IV, with absent or reduced interbarrel septa. In contrast, paroxetine treatment only changed the refinement, but not the formation, of barrel patterns of thalamocortical fibers since all the rows and most of the septa were conserved, with the barrel size decreased and the interbarrel septa enlarged.

Differences in the extent and time window of 5-HT uptake blockage between 5-HTT KO and paroxetine treatment may account for the differences in their effects on barrel pattern. Blockage of 5-HT uptake to thalamocortical fibers could fluctuate during the intervals between injections in paroxetine-treated pups, but 5-HT uptake to thalamocortical fibers is consistently absent in 5-HTT KO mice. Blockage of 5-HT uptake was carried out and influenced developmental events occurring only from P0-P8 (such as barrel pattern formation and refinement in somatosensory cortex) in paroxetine-treated animals, whereas 5-HTT KO mice, which have not had 5-HTT from the beginning of their life, may have developed unknown compensatory mechanisms. Nevertheless, the treatment of paroxetine reflects a possible clinical scenario of SSRI administration on brain development.

4.3. Possible mechanisms of desegregation of thalamic afferents to barrels

Paroxetine blocks the uptake of 5-HT, thereby increasing extracellular 5-HT concentration [14]. Increased extracellular 5-HT levels have been shown to affect the patterning of thalamocortical afferents and barrels in MAO-A KO mice [8]. Both 5-HTT KO mice [40] and mice treated with an MAO-A inhibitor from P0-P7 [42] demonstrate a very similar desegregation pattern of thalamocortical fibers to those of paroxetine-treated pups. Paroxetine at this dose (5 mg/kg/day, s.c. twice a day) may also increase extracellular noradrenaline concentrations [14]. However, it has been suggested that noradrenaline would be unlikely to play a primary role in the patterning of thalamocortical fibers and barrels since only inhibitors of 5-HT, but not inhibitors of catecholamine synthesis, could restore the barrel pattern in MAO-A KO and MAO-A inhibitor- treated mice [8,42]. Among all the 5-HT-associated molecules expressed in barrel field such as 5-HT transporters (5-HTT, $VMAT_2$, [24,25]) and receptors ($5\text{-}HT_{2A}$ [29] and $5\text{-}HT_{1B}$) in thalamocortical fibers, $5\text{-}HT_{1B}$ has been shown to play an important role in the segregation of thalamocortical fibers as well as mediating the effects of 5-HT on the segregation. Selective activation of $5\text{-}HT_{1B}$ receptors alters the segregation of thalamocortical fiber patches in a similar pattern to those seen in this study [46]. Desegregation in 5-HTT KOs and MAO-A KOs are normalized by additional $5\text{-}HT_{1B}$ receptor KO [40]. Therefore, the increased 5-HT levels in the extracellular space and the overactivation of $5\text{-}HT_{1B}$ receptors in thalamocortical fibers may contribute primarily to the desegregation of thalamocortical fibers in paroxetine-treated pups.

4.4. Possible mechanisms of the decreased size of thalamocortical fiber patches in paroxetine-treated pups

It has been reported that decreased barrel size is closely correlated with decreased brain weight caused by 5-HT-depleting agents, and the drug treatments that do not decrease brain weight do not affect the barrel sizes [31]. However, the decreased size of thalamocortical fiber

patches was most likely not due to the influences of paroxetine on general brain growth because the brain weight and the cortical area devoted to the representation of the caudal whiskers were normal in paroxetine-treated pups. Decreased cross-sectional areas of thalamocortical patches indicate the reduced arborization of thalamocortical terminals.

In further consideration of the mechanism, the decreased arborization of thalamocortical fiber patches in paroxetine-treated animals can not be explained by the increased extracellular 5-HT levels and subsequently increased activation of 5-HT_{1B} receptors, which have tropic effects on the development of these thalamocortical fibers and are associated with larger barrels than normal [27,28,42]. However, it is likely that the reduced arborization of thalamocortical fiber terminals may be related to the decreased internalization of 5-HT into these fibers. Both 5-HT depletion [3] and 5-HT uptake blockage in this study reduced the amount of 5-HT internalized into the developing thalamocortical fibers and decreased the size of these fibers. Moreover, MAO-A inhibition increases the uptake of 5-HT into thalamocortical fibers and also results in larger barrels [42]. Further experiments need to be done to investigate the effects of paroxetine on axon growth and arborization of cultured thalamic neurons during the first postnatal week.

Regarding to the differential sensitivity of regional barrel development, it has been suggested that the thalamocortical fibers projecting to rostral barrels are more vulnerable than those to caudal barrels because of a caudal-to-rostral developmental gradient [41,42]. This is well-reflected in our treatment paradigm; the severity of barrel abnormalities demonstrated a caudal-rostral gradation in that the more severe alterations of barrel morphometry occurred in caudal aspects [such as in row A] of PMBSF and ALBSF which showed desegregation as well as noticeable size reduction. The next most severe were the patches corresponding to the caudal long whiskers; B1-B3, C1-C3, D1-D4, and E1-E5 were reduced in size, while the relatively rostral whiskers, B4, C4 and D5, had fewer or no abnormalities in paroxetine-treated pups (Fig. 7a).

4.5. Topographic map of thalamocortical fiber patches corresponding to long caudal whiskers

Normal distance between [centers of] adjacent thalamocortical fiber patches with reduced patch size indicates two consequences-fewer thalamocortical connections in the barrel and enlargement of septa for these thalamocortical fiber patches corresponding to the caudal whiskers. Patches of thalamocortical fibers to C4 and D4 were closer to each other along the arc, which indicates possible additional alterations from sensitivity to discriminatory acuity. These topographical changes altered the somatosensory reception map.

4.6. Significance of the effects of paroxetine on the development of thalamocortical fibers to barrel cortex

It has been reported that chronic prenatal exposure to SSRIs do not change the growth, physical maturation [34] and major behaviors [10] in mouse offspring. In human prospective studies, maternal use of SSRIs was shown not to increase rates of major malformation, miscarriage, stillbirth [11,22,23] or affect the gross development of language, behavior and IQ [30]. Similarly, in our study, paroxetine treatment during the first postnatal week also did not change brain weight of rat pups. However, we report here that critical refinement and organization of barrel-like clusters of thalamocortical fibers were altered in paroxetine-treated pups at the stage of crucial somatosensory circuit formation. Thalamocortical fibers projecting to the barrel cortex relay important sensory information from one whisker to one corresponding barrel representation in cortex and the segregation of thalamocortical afferents is required for the establishment of this one-to-one functional relationship [13,43]. The desegregation and decreased size of thalamocortical afferents may indicate a less precise relay of topographic sensory information from those whiskers. The long-term consequences and the subtle changes in somatosensory sensitivity and acuity of children upon maternal SSRI intake during pregnancy remain to be investigated in humans. Cautions should also be taken that the use of alcohol or other drugs during pregnancy affecting the 5-HT levels may have consequence on sensory development [33].

In summary, the development of barrel-like clusters of thalamocortical fibers or the neural circuitry in rat somatosensory cortex was affected by exposure to a SSRI, paroxetine, during the first postnatal week, which may correspond to the third trimester of human pregnancy. Our study warrants further investigation that precautions might be taken when prescribing SSRIs for pregnant woman.

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