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Reproductive experience modified dendritic spines on cortical pyramidal neurons to enhance sensory perception and spatial learning in rats

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Abstract: Behavioral adaptations during motherhood are aimed at increasing reproductive success. Alterations of hormones during motherhood could trigger brain morphological changes to underlie behavioral alterations. Here we investigated whether motherhood changes a rat's sensory perception and spatial memory in conjunction with cortical neuronal structural changes. Female rats of different statuses, including virgin, pregnant, lactating, and primiparous rats were studied. Behavioral test showed that the lactating rats were most sensitive to heat, while rats with motherhood and reproduction experience outperformed virgin rats in a water maze task. By intracellular dye injection and computer-assisted 3-dimensional reconstruction, the dendritic arbors and spines of the layer III and V pyramidal neurons of the somatosensory cortex and CA1 hippocampal pyramidal neurons were revealed for closer analysis. The results showed that motherhood and reproductive experience increased dendritic spines but not arbors or the lengths of the layer III and V pyramidal neurons of the somatosensory cortex and CA1 hippocampal pyramidal neurons. In addition, lactating rats had a higher incidence of spines than pregnant or primiparous rats. The increase of dendritic spines was coupled with increased expression of the glutamatergic postsynaptic marker protein (PSD-95), especially in lactating rats. On the basis of the present results, it is concluded that motherhood enhanced rat sensory perception and spatial memory and was accompanied by increases in dendritic spines on output neurons of the somatosensory cortex and CA1 hippocampus. The effect was sustained for at least 6 weeks after the weaning of the pups.

Key words: cerebral cortex, dendritic spine, hippocampus, lactation, reproductive experience

Introduction

Motherhood and the postpartum period are challenging times for females. In adapting to look after offspring for ensuring reproductive success, females undergo a

series of hormonal, neurological, and behavioral changes [30]. Among them, fluctuation of hormonal levels during motherhood is believed to lead to plasticity of the maternal brain and behavioral alternations. At the gross level, an imaging study of the maternal human reported

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decreases in brain sizes throughout pregnancy and increases after delivery [43]. In experimental animals, maternal rats were found to have an altered hippocampus size [12] and thickness of the cerebral cortex [17].

Increases in serum estradiol, which increases approximately 3-fold from proestrus to day 20 of pregnancy [2], are known to affect maternal behaviors and are directly related to brain areas including the medial preoptic area (mPOA), amygdala, parietal cortex, prefrontal cortex [10], hypothalamus [11], and olfactory bulbs [6]. In addition, changes in estradiol also appear to affect brain regions that are not commonly associated with maternal behaviors, such as the hippocampus. At the cellular level, estradiol promotes the formation of new dendritic spines in hippocampal neurons [24, 38, 68] and increases the hippocampal neuronal apical dendritic spine density [14, 22, 24, 67], neurogenesis in the dentate gyrus [57], and long-term potentiation [64]. The other important hormone of reproduction, progesterone, was also found to increase the density of dendritic spines on the apical dendrites of hippocampal neurons [67] and to provide neuroprotection [55]. The parturition hormone oxytocin was found to enhance cell proliferation in the dentate gyrus [28] and hippocampal long-term potentiation [60]. The postpartum period enhances the dendritic spine density on pyramidal neurons of the medial prefrontal cortex (mPFC) in mother rats [31]. The lactating hormone prolactin on the other hand, mediates hippocampal neurogenesis [61] and provides neuroprotection [59].

Dendritic spines are small dynamic protrusions of neuronal dendrites that function as postsynaptic components of the majority of excitatory synapses, and they have been widely considered to be the repositories of long-term memory [54]. Many studies on dendritic spines have focused on hippocampal pyramidal neurons. The CA1 pyramidal neuronal dendritic spine density was first reported to fluctuate throughout the estrus cycle with the level of estradiol in female rats [65]. However, the fluctuation of hormone levels throughout the estrus cycle is not as great as that throughout motherhood. Hippocampal CA1 dendritic spines were later found to be even higher during late pregnancy and lactation than during the estrus cycle. This is consistent with the notion that estradiol and progesterone are capable of regulating hippocampal neuronal dendritic spines [22, 24]. Another interesting finding is that the profuseness and length of the dendrites of CA1 and CA3 hippocampal neurons

were reported to have decreased 1 month after delivery [47], suggesting post-motherhood brain plasticity.

Unlike the hippocampus and areas of the brain directly related to reproduction, little is known about whether and how sex hormones and motherhood affect the primary cortex. Martinez-Gomez's study showed that the female reproductive cycle may modify responsiveness to noxious stimuli [35]. Our study found that estradiol in the rat somatosensory cortex modulates the dendritic spines, but not dendritic arbors, of its output neurons, namely layer III and V pyramidal neurons [3]. In addition, progesterone also regulates dendritic spines on these neurons, as treating ovariectomized rats with progesterone alone or with estradiol rescued the loss of dendritic spines [3, 63]. It remains to be determined how motherhood affects the primary cortical neurons.

In this study, we explored whether motherhood affects layer III and V pyramidal neurons of the somatosensory cortex and pyramidal neurons of the CA1 hippocampus. Rats during pregnancy, lactation, and 6 weeks after weaning (primipara) were studied, with virgin rats as the control. Intracellular dye injection was used to reveal the dendritic arbors of the studied neurons for analyses of their length and dendritic spine density. A hot plate test and Morris water maze task were used to assess alterations of sensory perception and spatial memory, respectively.

Materials and Methods

Forty-six 3-month-old female Sprague-Dawley rats were used. Rats were caged individually with food and water *ad libitum* in a temperature ($24 \pm 1^\circ\text{C}$) and humidity-controlled room with a 12-h light-dark cycle. Experiments were approved by the Animal Care and Use Committee of the National Chung-Hsing University under guidelines of the National Science Council of Taiwan.

Motherhood and timing of behavioral tests

Ten female rats were subjected to a complete round of motherhood, from mating, pregnancy, and lactation to pup weaning. Four sessions of behavior tests were conducted on these rats when virgin, on day 16–18 of pregnancy (P16-P18), on postpartum day 11–13 (PP11-PP13) during lactation, and at 6 weeks after pup weaning (approximately postpartum day 63 depending on the

determination of proestrus). Each session consisted of a water maze task followed by a hot plate test daily.

Water maze task

A modified Morris water maze was adopted. A white 185-cm diameter pool with a water depth of 24 cm was placed in a sound-attenuated room. Numerous distant visual cues (cabinet, refrigerator and biosafety cabinet, door) were scattered around the room, and 3 close visual cues (triangle, round and square cardboards) were located at the edge of the pool. A round transparent platform was placed 2 cm below the surface of the water. After the virgin rats underwent the first test session, they were mated with sexually experienced male rats. The day of appearance of a vaginal plug was designated as day 1 of pregnancy (P1). The second water maze test session was conducted on P16-P18 of pregnancy. The third test session was performed on the 11th to 13th postpartum day (PP11-PP13) in lactating rats. The last water maze test session was conducted on rats 6 weeks after weaning (primipara), mostly on PP63 for most pups weaned on PP21. The visual cues and platform location were rearranged in the first trial of each session. In each trial of the water maze test, rats were randomly placed into different quadrants of the pool. Latency in locating the hidden platform was recorded. The tested animals were guided to the platform when they failed to locate the platform within 5 min. In this case, a maximum latency of 5 min was recorded. All rats were allowed to remain on the platform for 30 s and were then returned to the cage. Two trials were conducted for each animal per day. Each session involved trials on 3 consecutive days.

To prevent the carrying over of previous experience due to rearranging cues, platform location, starting point, and the experimenter's position at the beginning of each session of the Morris water maze test, 6 additional virgin rats were subjected to two sessions of 3-day Morris water maze tasks, 20 days apart, as described above. Our tests showed that there was no difference in performance between these two sessions (Fig. 3B). Thus rearranging visual cues before each session is an effective means of resetting the water maze as a new task.

Hot plate test

A slightly adapted hot plate test, as described originally by Eddy and Liembach [9], was used to evaluate the effect of motherhood and reproductive experience

on sensory perception. The test was conducted 2 h after the water maze task each day to ensure that the rats were dry. One hot plate test was performed per day. Briefly, the tested device (Ugo Basile, Comerio, VA, Italy) was preheated to 48°C. Rats were then gently placed on the plate, and a timer was triggered by the experimenter once all their paws were in contact with the hot plate. The rats were then monitored, and the latency time of foot lick was recorded. Rats were removed from the hot plate if they did not respond in 120 s, and a maximum latency of 120 s was recorded. The foot lick latency of the same rat within sessions was averaged and normalized to that of virgin rats and analyzed.

Intracellular dye injection and subsequent conversion of the injected dye

Five rats each at the virgin, pregnancy (day 18 of pregnancy), lactation, and primipara stages were processed for intracellular dye injection. Litters of the lactation rats were immediately assigned to foster females after the sacrifice of their biological mothers. Virgin and primiparous rats were confirmed to be at proestrus by vaginal smear.

Upon sacrifice, rats were deeply anesthetized with ketamine and xylazine (8 mg ketamine and 1 mg xylazine/100 g body weight) and transcardially perfused with 100 ml of saline followed by 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) for 30 min, and this allowed the cellular membrane in brain slices to maintain a fluid state for electrode penetration and sealing. The brains were then removed and sectioned in PB with a vibratome into 350- μ m-thick coronal slices as described previously [3–5, 62]. Slices containing the somatosensory cortex and hippocampal region were collected in PB. They were then immersed in 10^{-7} M 4,6-diamidino-2-phenyl-indole (DAPI; Sigma-Aldrich, St Louis, MO, USA) PB solution for 30 min so that cell nuclei could be visualized under the same fluorescence filter set (390–420, FT 425, LP 450) that revealed the fluorescence of the intracellular dye Lucifer yellow (LY, Sigma-Aldrich; 4% in water). For injection, a DAPI-treated slice was placed in a well filled with 0.1 M PB on the stage of an epifluorescence microscope (Olympus BX51). An intracellular micropipette filled with LY solution was mounted on a 3-axial hydraulic micromanipulator (Narishige, Tokyo, Japan), and a long working distance objective lens ($\times 20$) was used to facilitate the selection of pyramidal neurons of the studied cortex. An intracellular

amplifier (Axoclamp-IIB) was used to generate the constant negative current (0.2 nA) for injecting LY until terminal dendrites fluoresced brightly (layer III or CA1 pyramidal neurons for approximately 5–6 min; layer V pyramidal neurons for approximately 10–12 min). The injected slice was then removed, washed with PB, and postfixed in 4% paraformaldehyde in PB overnight. To convert LY into non-fading material, injected slices were cyroprotected in 30% sucrose and resectioned into 60- μm -thick serial sections with a cryostat (CM1850, Leica, Nussloch, Germany). Sections were treated with 1% H_2O_2 in PB for 60 min, followed by 10% bovine serum albumin and 1% Triton-X in 10 mM phosphate-buffered saline (PBS) for an hour. They were then incubated with solution containing biotinylated rabbit anti-LY (1:200; Molecular Probes, Eugene, OR, USA) in PBS for 18 h at 4°C. After rinses in PBS, sections were treated with avidin-biotin HRP reagent (Vector, Burlingame, CA, USA) for 1 h at room temperature. At the end, they were reacted with 3–3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) and 0.01% H_2O_2 in 0.05 M Tris buffer. Reacted sections were mounted on gelatin-coated slides, dehydrated, cleared, and coverslipped.

Three-dimensional reconstruction of the injected pyramidal cells

The dendritic arbors of 6 layer III and 6 layer V pyramidal neurons of the somatosensory cortex and 6 CA1 pyramidal neurons of the hippocampus of each rat were reconstructed 3-dimensionally with NeuroLucida (MicroBrightField, Williston, VT, USA). The dendrogram [5, 63] and the length and number of terminal ends of the dendrites of each reconstructed neuron were analyzed with NeuroExplorer (MicroBrightField). The value of each parameter for each animal is the mean of the 6 neurons analyzed. An example of reconstruction of a layer III pyramidal neuron of the somatosensory cortex is shown in Fig. 1. Soma and dendrites were darkly stained after immunoconversion of the injected dye (Fig. 1A). Following 3-dimensional reconstruction with NeuroLucida, a complete dendritic arbor of a neuron could be routinely represented (Fig. 1A'). The dendritic arbor of the pyramidal neuron allowed subsequent comparisons of dendritic parameters between experimental groups.

A $\times 100$ oil-immersion objective lens was used to analyze the density of dendritic spines on somatosen-

sory cortical and hippocampal pyramidal neurons. The proximal and distal basal dendrites were defined as segments located 25–75 μm and 100–150 μm from the soma for layer III pyramidal neurons and 50–100 μm and 150–200 μm from the soma for layer V pyramidal neurons, respectively. For both neurons, the first and second branches of the apical trunk were defined as proximal apical dendrites, and the terminal dendrites of the apical tuft were defined as distal apical dendrites. For hippocampal CA1 pyramidal neurons, proximal and distal apical dendrites were those in the stratum radiatum and stratum lacunosum moleculare, while basal dendrite referred to those confined to the stratum oriens. To analyze dendritic spines in each neuron, 6 representative pieces of each category of the dendritic segments were reconstructed, and the spine density was evaluated. In each cell, the spine density of each studied segment was the mean of 6 corresponding segments measured. The spine density of each pyramidal neurons of each animal was the mean of the 6 corresponding neurons studied in each animal.

In addition, dendritic spines were sorted based on their morphologies into 3 types [50] for further analysis of their changes: type 1, consisting of stubby spines that were small swelling protrusions of the dendritic trunk lacking a clear stalk; type 2, consisting of mushroom spines that had a stalk and a mushroom-shaped head, with the length of the stalk shorter than the diameter of the head; type 3, consisting of thin spines comprised of a thin and elongated stalk and a relatively small head. Under $100\times$ objective lens, the DAB dark brown staining also revealed the great detail morphology of individual spine. According to their morphological appearance, spines can be classified into stubby (type 1), mushroom (type 2), and thin (type 3) spines (Fig. 1B and 1B').

Western blotting of PSD-95

To find out whether changes in spine density represent excitatory synaptic changes, the expression of PSD-95, a glutamatergic postsynaptic marker involved in spine maturation and clustering of synaptic signaling proteins, was evaluated. The somatosensory cortices and hippocampi of virgin, pregnant, lactating, and primiparous rats ($n=5$ each) were harvested and homogenized in Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). Homogenized tissues were kept in ice for 20 min followed by centrifugation at 12,000 g for

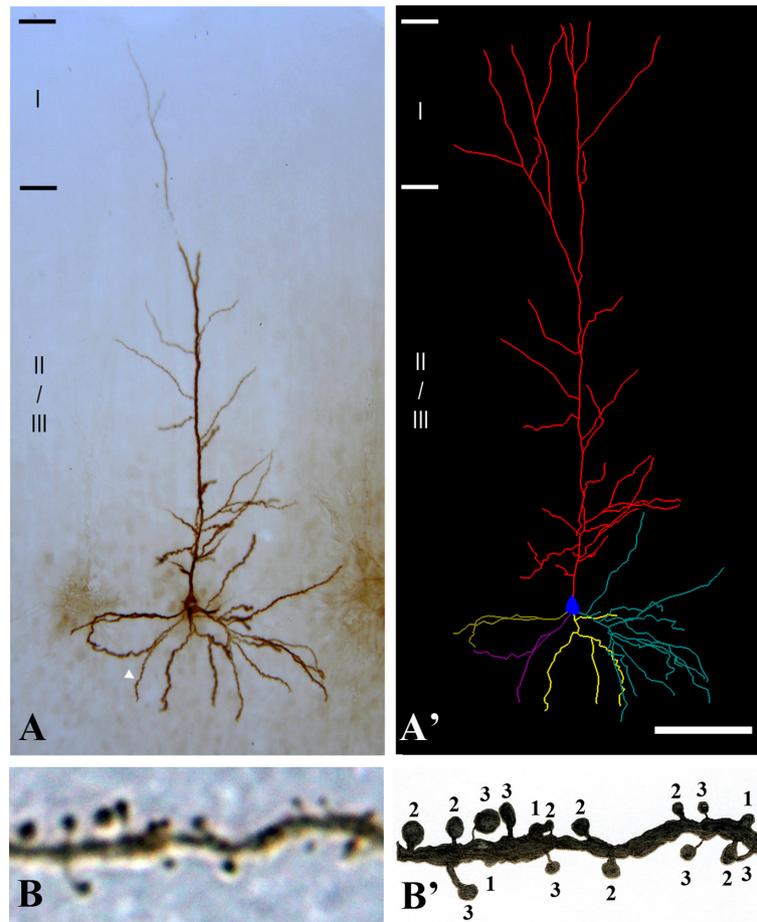


Fig. 1. A representative intracellular dye-filled layer III pyramidal neuron of the primary somatosensory cortex of a pregnant rat. (A) Micrograph of the neuron from one of the 60- μm -thick sections of the brain slice in which the neuron was dye filled. (A') The neuron after 3-dimensional reconstruction using the series of sections of the brain slice that contained the neuron. Roman numerals and horizontal bars in the drawing mark and demarcate cortical layers, respectively. Branches of the same dendritic trunk are shown in one color. B and B' was a micrograph and camera lucida drawing of a dendritic segment at about the location indicated by the arrowhead in A. B' illustrates the spine density and classifying the spine into three different types (B') by adjusting the focus while analyzing. Type 1 (stubby), type 2 (mushroom), and type 3 (thin) spines are labeled. Scale bar=100 μm for A and 5 μm for B.

20 min to extract total protein. The concentration of the extracted total protein was determined utilizing a Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Proteins were resolved with 10% polyacrylamide gels containing sodium dodecyl sulfate. Resolved proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad), the membrane was cut into two portions, and both portions were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 3% skim milk for 1 h, followed by overnight incubation at 4°C in

mouse anti-PSD-95 (1:500, Chemicon, Temecula, CA, USA) or mouse anti-GAPDH (1:1,000, Chemicon) in TBST, respectively. They were then incubated with an HRP-conjugated anti-mouse antibody in TBST for 1 h (1:5,000, Jackson ImmunoResearch, West Grove, PA, USA), developed with Enhanced Chemiluminescence Western Blotting Substrate (Thermo scientific), and finally imaged with an LAS-3000 luminescence image analyzer (Fujifilm, Tokyo, Japan). The optical densities of visualized bands were analyzed and normalized to

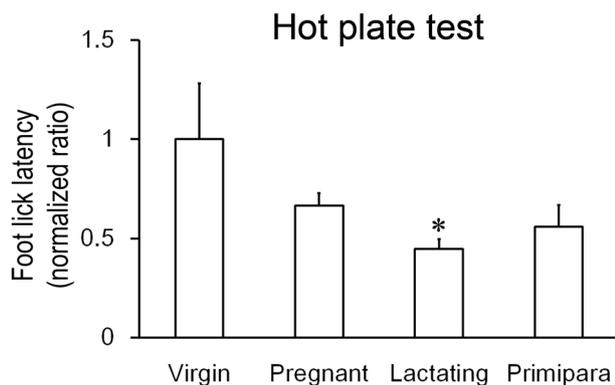


Fig. 2. Effects of motherhood and reproductive experience on sensory perception. Rats were tested with a 48°C hot plate. The latency of foot lick in virgin, pregnant, lactating, and primipara rats was normalized to that of the virgin rats. * $P < 0.05$ between the indicated rats and virgin rats

GAPDH with ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were expressed as the mean \pm SE unless otherwise indicated. In between-group comparisons, we used one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test for comparison of groups for the hot plate test, dendritic arbors, spine density, and Western blotting. The water maze task was analyzed using two-way ANOVA followed by Tukey's *post hoc* comparisons.

Results

Motherhood enhanced heat sensitivity and spatial memory

Rats responded to mild heat stimulation (48°C) by licking their foot. Pregnant, lactating, and primiparous rats responded with shorter latencies, 67%, 45%, and 56% of that of the virgin rats, respectively, although only the reduction of the lactation group reached statistical significance (Fig. 2, $F = 7.912$, $P < 0.05$).

The swimming time shortened gradually over the 3 days of testing in all groups (Fig. 3A), indicating gradual spatial memory acquisition in all animals. Significant differences between different days of testing ($F = 50.67$, $P < 0.001$) and among groups ($F = 7.87$, $P < 0.001$) were revealed following 2-way ANOVA. Post hoc comparisons of the escape times of the pregnant, lactating, and primiparous rats indicated that they required shorter

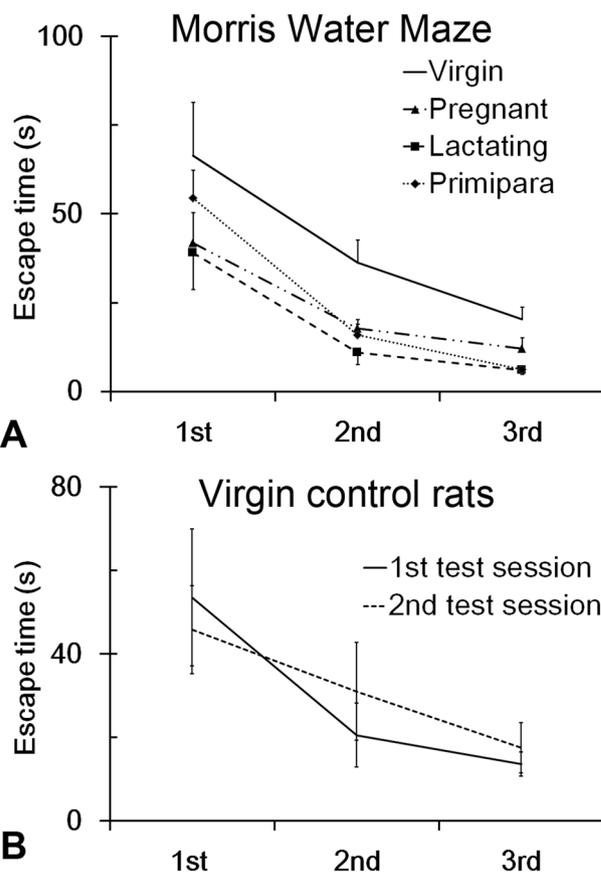


Fig. 3. Results of the Morris water maze task. A group of rats was subjected to one 3-day session of testing, with 1 trial per day, in each of the 4 statuses, i.e., virgin, pregnant, lactating, and primipara. The escape latencies for each session of the 3 tests are plotted in A. Another group of virgin rats was subjected to 2 sessions of tests separated by 20 days with visual cues rearranged at the beginning of the second session (B). There was no apparent carrying over effect of previous water maze test experience.

amounts of time to locate the hidden platform than the virgin rats. There were no significant interactions between day of testing and groups ($F = 2.45$, $P = 0.85$).

Motherhood altered dendritic spines on somatosensory cortical pyramidal neurons

Motherhood and reproductive experience were found not to affect the apparent shape of the dendritic arbor (details not shown), dendrogram (details not shown), number of terminal ends (Fig. 4A, $F = 1.14$, $P = 0.35$ for the basal dendrite; $F = 0.53$, $P = 0.63$ for the apical dendrite; $F = 1.11$, $P = 0.34$ for the total dendrite), or dendritic length (Fig. 4B, $F = 2.35$, $P = 0.35$ for the basal dendrite; $F = 0.63$, $P = 0.43$ for the apical dendrite; $F = 0.95$,

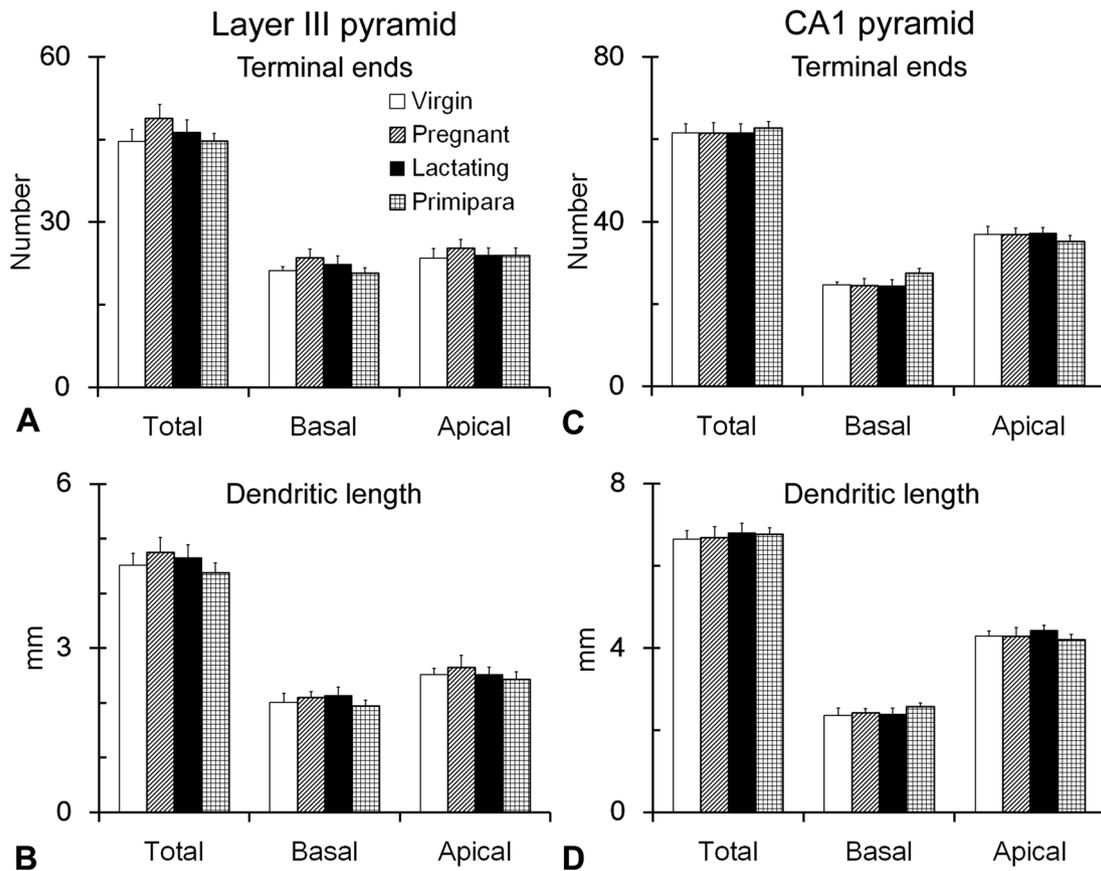


Fig. 4. Effects of motherhood and reproductive experience on the dendritic arbors of cortical and hippocampal neurons. Analyses of the numbers of terminal ends (A and C), and dendritic length (B and D), of the layer III somatosensory cortical pyramidal neurons and CA1 hippocampal pyramidal neurons in the 4 groups of rats are illustrated on the left and right respectively. Numbers of terminal ends and lengths of the basal, apical, and total dendritic arbors were derived from 3-dimensionally reconstructed neurons.

$P=0.46$ for the total dendrite).

We then looked at dendritic spines on these neurons (Fig. 5A). Our analyses show that in layer III pyramidal neurons, the spine density on all dendrites, except proximal apical dendrites, were significantly increased during pregnancy and that all of them were further significantly increased during lactation and returned to (proximal apical and distal basal dendrites) or remained somewhat higher than individual virgin control dendrites (distal apical and proximal basal dendrites) 6 weeks after the pups were weaned (Fig. 5B, left half). For layer V pyramidal neurons, pregnancy significantly increased dendritic spines on distal apical and basal dendrites, lactation increased dendritic spines on all 4 segments dramatically to the highest level, and dendritic spines on all dendrites, except distal basal dendrites, returned to the virgin level 6 weeks after pups were weaned (Fig. 5B,

right half).

When dendritic spines were typed based on morphology, types 2 spines on all segments of the apical and basal dendrites of layer III and layer V pyramidal neurons were significantly increased throughout motherhood and in primipara rats (Fig. 5B). The number of type 3 dendritic spines first decreased during pregnancy and then increased dramatically to significantly higher than the virgin level by lactation, returning to the virgin levels 6 weeks after pups were weaned (Fig. 5B). The numbers of type 1 spines remained low throughout motherhood and in primipara rats.

Motherhood increased dendritic spines on hippocampal CA1 pyramidal neurons

Like the somatosensory cortex, motherhood and reproductive experience did not alter the appearance of the

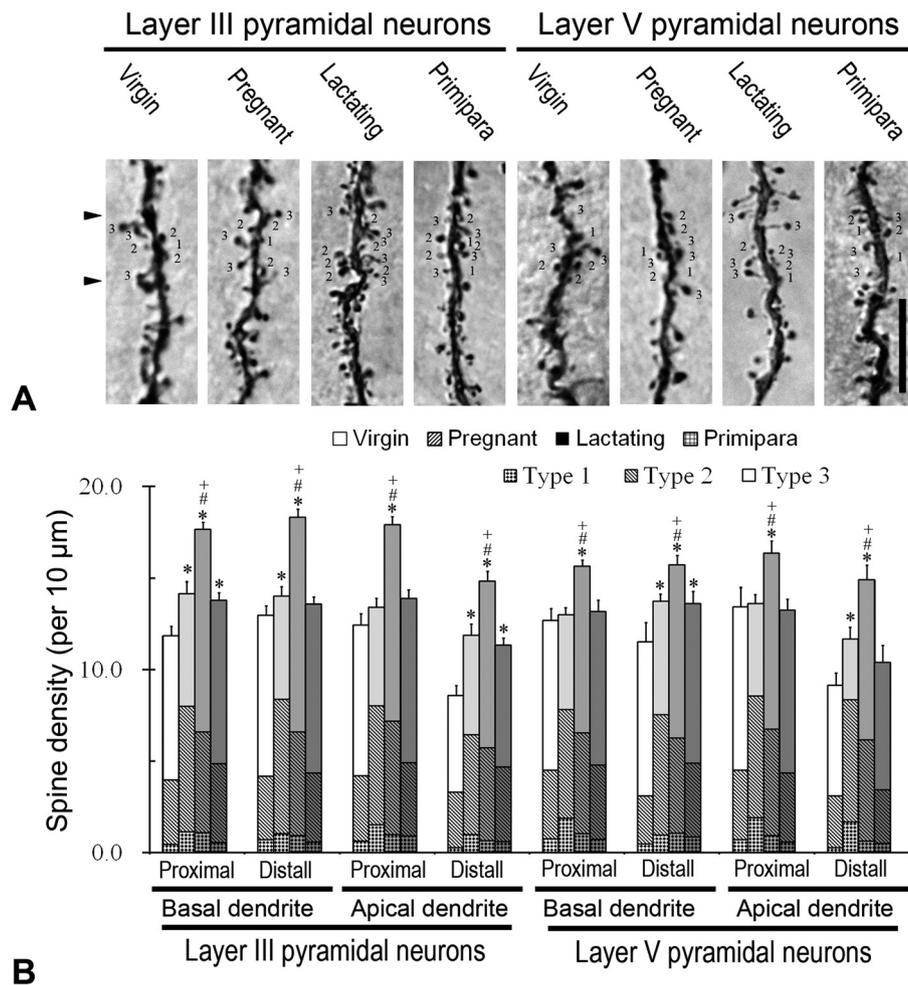


Fig. 5. Effects of motherhood and reproductive experience on spine density of layer III and V somatosensory cortical pyramidal neurons. High-power micrographs of representative proximal apical dendrites of virgin, pregnant, lactating and primipara rats are illustrated in A. Those from layer III pyramidal neurons are shown on the left, while layer V pyramidal neurons are shown on the right. The spine types of dendritic segments are labeled with numbers between the arrowheads in A. Changes in spine density and type in all 4 dendritic segments analyzed are plotted in B with the layer III pyramid on the left and layer V pyramid on the right. * $P < 0.05$ between the indicated rats and virgin rats. # $P < 0.05$ between the indicated rats and pregnant rats. + $P < 0.05$ between the indicated rats and primiparous rats. Scale bar=10 μm for all micrographs in A.

dendritic arbor (not shown), number of dendritic terminals (Fig. 4C, $F=1.04$, $P=0.38$ for the basal dendrite; $F=0.3$, $P=0.83$ for the apical dendrite; $F=0.91$, $P=0.44$ for the total dendrite), or dendritic length (Fig. 4D, $F=0.39$, $P=0.77$ for the basal dendrite; $F=0.33$, $P=0.80$ for the apical dendrite; $F=0.51$, $P=0.68$ for the total dendrite). On all three dendritic segments analyzed, spine density increased significantly during pregnancy, further increased to higher than the pregnancy level during lactation, and decreased slightly but remained higher than in the virgin and pregnant rats 6 weeks after weaning of

pups (Fig. 6).

The number of type 2 dendritic spines on CA1 hippocampal neurons was found to be increased during pregnancy, to be further increased during lactation, and to have returned to the pregnancy level in primipara rats (Fig. 6). The numbers of type 3 spines on all three dendritic segments showed a delayed increase. For apical dendrites, spine density became significantly increased by lactation and remained high in primipara rats. For basal dendrites, the spine density became significantly increased until the primipara period. The number of type

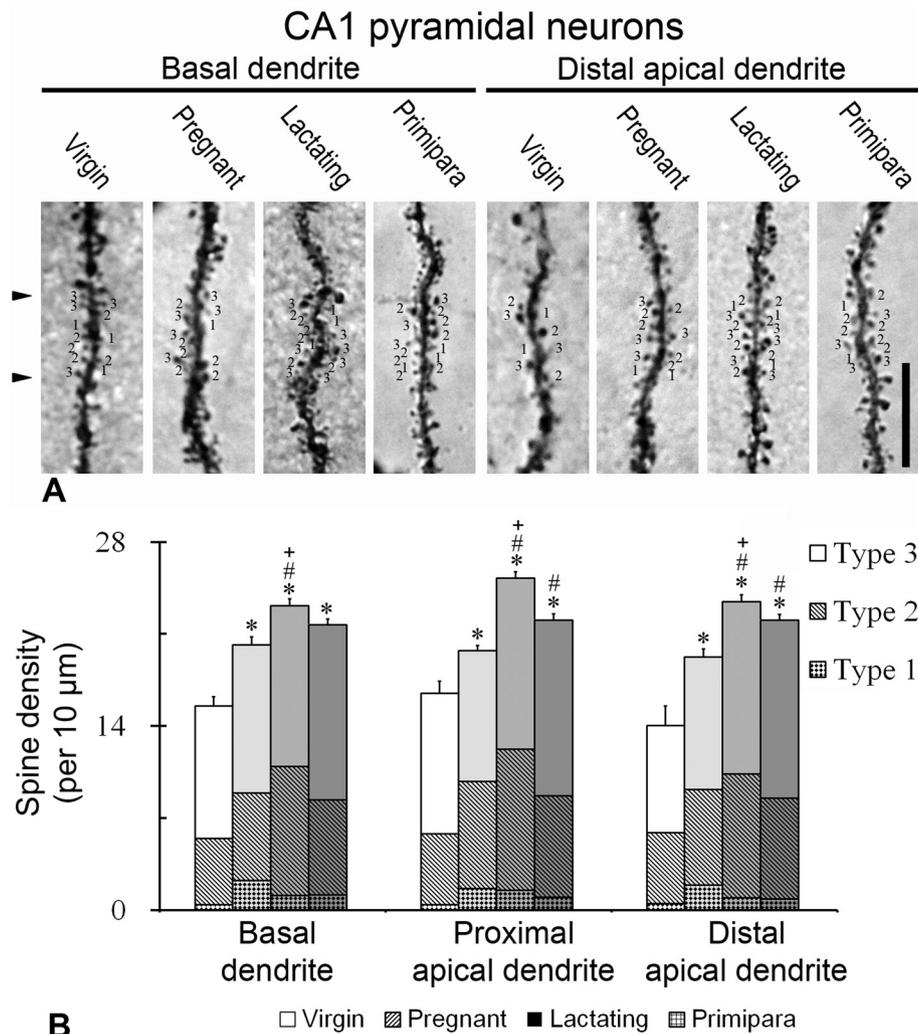


Fig. 6. Effects of motherhood and reproductive experience on dendritic spine density of CA1 hippocampal pyramidal neurons. (A) Micrographs of representative basal dendrites and distal apical dendrites of CA1 pyramidal neurons of virgin, pregnant, lactating, and primiparous rats. The spine types of dendritic segments are labeled with numbers between the arrowheads in A. Analyses of the changes in spine density and type are plotted in B. Pregnant, lactating, and primiparous rats had more spines than virgin rats. * $P < 0.05$ between the indicated rats and virgin rats. # $P < 0.05$ between the indicated rats and pregnant rats. + $P < 0.05$ between the indicated rats and primiparous rats. Scale bar = 20 μm for all micrographs in A.

1 spines remained relatively low throughout motherhood and the primipara period (Fig. 6).

Increase in PSD-95 expression accompanied dendritic spine increases

PSD-95 expression in the somatosensory cortex and hippocampus was significantly increased with motherhood and reproductive experience (Fig. 7). In the somatosensory cortex, PSD-95 expression during lactation was markedly increased to a level higher than at other stages of motherhood (Fig. 7A).

Discussion

We demonstrated in this study that motherhood altered the density and shape of dendritic spines on primary somatosensory cortical neurons. Concomitantly, the heat sensitivity of the mother rats was enhanced. In addition, motherhood also increased the density of dendritic spines on hippocampal CA1 pyramidal neurons with a concomitant enhancement of spatial memory. Both structural and behavioral enhancements were sustained, but

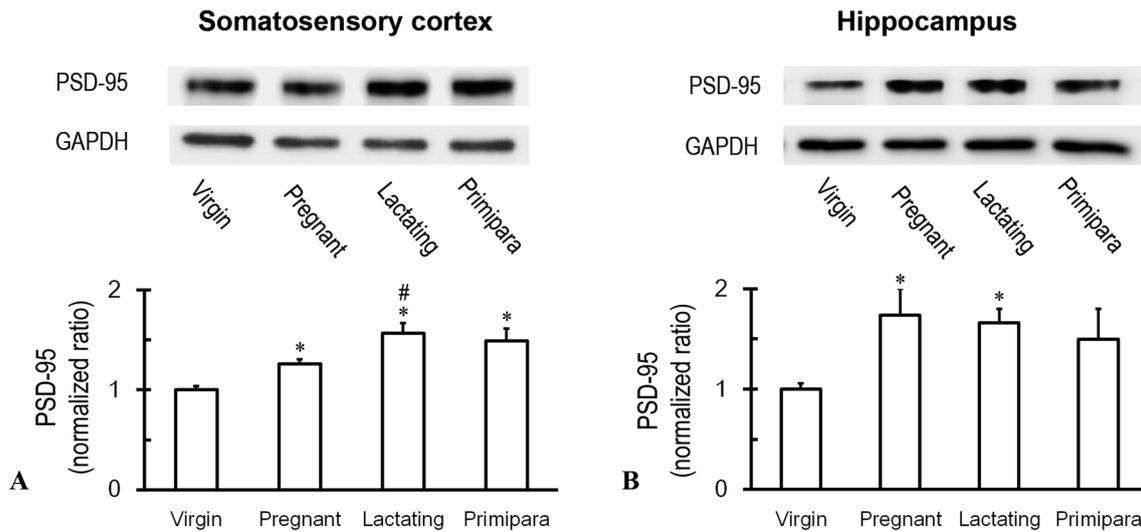


Fig. 7. Effects of motherhood and reproductive experience on the expression of the glutamatergic postsynaptic marker protein PSD-95. Representative western blot of PSD-95 in the primary somatosensory cortex (A) and hippocampus (B) of virgin, pregnant, lactating, and primiparous rats are illustrated in the upper halves. The expression was normalized to that of the corresponding internal control (GAPDH) and plotted onto the bar graph at the bottom. * $P < 0.05$ between the indicated rats and virgin rats. # $P < 0.05$ between the indicated rats and pregnant rats.

at a reduced level, after weaning of the pups. These findings provide a basis for central neuronal structural plasticity for the enhancement of maternal performance during motherhood and afterward.

Experimental design

Reproduction-related hormones have been regarded as dominant factors affecting the body and behavior during motherhood [3, 13, 30, 31, 66, 67]. In the present experimental design, a precise timing was selected for examination of rats at various stages. In the rat, the spine density of hippocampal CA1 pyramidal neurons [14, 65] and layer III and layer V somatosensory cortical pyramidal neurons [3] undergoes a cyclic fluctuation during the estrous cycle, so we used virgin rats at proestrus to avoid the influence of extra factors. For the effect of pregnancy, we examined P18 rats because the peripheral estrogen increases from P1 to P21, while peripheral progesterone starts to increase on P1, peaking on P18 and subsiding after P19 [37, 49, 58]. Thus P18 appears to be the optimal timing for studying the combined effect of estrogen and progesterone in pregnant rats, as both hormones affect the cortical neuronal dendritic spines [3]. To study the effect of lactation, we focused on the timing of changes in prolactin and oxytocin, as they both are the major hormones during this period. Prolactin is known to be at its highest level on PP13 and

to decrease thereafter in lactating rats [15]. On the other hand, intracerebral oxytocin is constantly maintained at a high level as long as rats receive nipple suckling stimulation [11]. We therefore chose to study PP13 lactating rats. Lastly, to find out whether the influences of motherhood's persist after pup weaning and after the estrus cycle had been restored, we examined primiparous rats 6 weeks after weaning. These rats were confirmed to be in proestrus before examination so that the data collected could be compared with those of the virgin rats, which were examined during this phase of the estrous cycle.

The association of dendritic alterations with behavioral changes

The rats that experienced motherhood were found, especially during lactation, to be more sensitive to heat and to have better spatial memory learning than virgin rats. During lactation, total dendritic spines on all segments of layer III and V somatosensory cortical pyramidal neurons were significantly increased compared with those of the virgin rats. A similar pattern of spine increase was identified in the hippocampal CA1 pyramidal neurons. Spine density increases were associated with increased glutamatergic postsynaptic marker protein PSD-95 expression, suggesting augmentation of excitatory inputs to these neurons. The above may be the underlying

ing structural basis for the enhancement of heat sensitivity and spatial memory learning. Such a notion would be in line with the positive correlation demonstrated earlier between neuronal dendritic spine density and spatial memory in the hippocampus [29, 39], olfactory learning in the piriform cortex [25], and recognition memory in the prefrontal cortex [62]. Although only the somatosensory cortex, the main area for body sensation, was explored in this study, we believe that most perception cortices, including olfactory, visual and auditory cortices, likely exhibit similar changes. These CNS neuronal changes could be the structural substrate for the behavioral adaptation of mothers, especially during lactation. These sensory inputs would provide rats who experience motherhood with higher sensitivity to the environment and acuteness in sensing the conditions and needs of pups and in searching for foods quickly so that the pups would not be left unattended or worse exposed to danger.

Dendritic spines are usually distinguished into 3 morphological types [50] reflecting their stability, maturity, and synaptic strength. Motherhood caused an apparent pattern of spine subtype changes in both layer III and layer V somatosensory cortical and CA1 hippocampal pyramidal neurons. Functionally, type 2 spines are believed to be the most mature and stable spines with the strongest synaptic strength [1, 41]. A larger spine head was reported to have significantly larger postsynaptic densities [53] that anchored more α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [36, 42, 56]. More AMPA receptors are associated with stronger synaptic strength and are essential for long-term potentiation [19, 33, 34, 44]; a deficiency on the other hand is associated with impaired spatial working memory [51]. Regarding type 3 dendritic spines, they are clearly the most numerous on the neurons studied. Most of them have a head and a long thin neck. They were reported to have smaller postsynaptic densities and more N-methyl-D-aspartate (NMDA) receptors [1], which upon activation could cause either rapid spine enlargement [32] or even retraction [16]. This is consistent with reports indicating that the type 3 spine is less stable than the type 2 spine in both the hippocampus [45] and neocortex [20]. The delayed large-scale increase in type 3 dendritic spines specifically during lactation further supports that they are the most plastic among the 3 types and endow pyramidal neurons a highly enhanced functional status that could have contributed to heat sensitiv-

ity and spatial memory learning being greatest at this stage. The above arguments are consistent with the finding that in the prefrontal cortex, type 3 spines were specifically increased in estrogen-treated ovariectomized monkeys that showed restored cognitive function [18], and on the other hand were decreased in age-related cognitively impaired monkeys [8]. In our results, the frequency of type 1 spines remained relatively unaltered. Type 2 spines quickly increased and peaked at pregnancy, while type 3 spines decreased or stayed roughly unchanged during pregnancy and then peaked sharply at lactation. These changes seemed to be in line with the inferences that mushroom spines are more stable, mature, and “memory” spines [1, 21, 40]. We speculate that increases in the density of type 2 spines in somatosensory cortical and CA1 hippocampal pyramidal neurons during pregnancy and lactation are likely to be linked to the enhanced sensory perception and spatial learning performances.

Differential persistence of motherhood's effects on CNS neurons after pup weaning

The persistence of motherhood's effects after pup weaning is deemed beneficiary, as it is likely to improve breeding success. As earlier studies, hyperalgesia was demonstrated during most of pregnancy, and pregnant rats were shown to have significantly lower pain thresholds than control rats. Pain thresholds were also significantly lower throughout the nursing period but increased significantly when dams were separated from their litters and subsequently returned to baseline values [7, 35]. A recent study showed that nursing-induced rats modulate the expression of glutamic acid decarboxylase and the NR2A subunit of NMDA that mainly encourage reshaping of cortical neuron receptive fields in the primary somatosensory cortex [52]. In addition, numerous earlier studies found that primiparous rats perform significantly better than age-matched virgin rats in water, dry land, and radial arm maze tasks for up to one and a half years after weaning [23, 26, 27, 46, 48]. In this study, primiparous rats 6 weeks after weaning showed enhanced spatial memory and ambiguously enhanced heat sensitivity. This is consistent with our anatomical finding of a protracted increase in spines over the entire dendritic arbor of hippocampal CA1 pyramidal neurons (Fig. 6). The majority of these persistently increased spine types were types 2 and 3. On the other hand, the increase in dendritic spines on layer III and V somatosensory corti-

cal pyramidal neurons during motherhood seems to be less robust. The increases of about half of the dendritic segments persisted, while the other half returned to the level of the virgin rats after pup weaning (Fig. 5).

Using fixed tissue intracellular dye injection and 3-dimensional reconstruction methods, we demonstrated that rats that experienced motherhood exhibited altered dendritic spines but not arbors on the primary somatosensory cortical and CA1 hippocampal pyramidal neurons. In this connection, it is striking that heat sensitivity and spatial memory were enhanced. These changes appeared to peak during lactation and persisted especially in the hippocampus after weaning of pups. The concurrent correlational behavioral and morphological changes suggest that alterations of dendritic structures are putative anatomical substrates underlying the behavioral adaptations.

Conflict of Interest

The authors declare that there are no conflicts of interest with regard to the organizations that sponsored the research.

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