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Effects of multiparity on recognition memory, monoaminergic neurotransmitters, and brain-derived neurotrophic factor (BDNF)

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Abstract

Recognition memory and anxiety were examined in nulliparous (NP: 0 litters) and multiparous (MP: 5–6 litters) middle-aged female rats (12 months old) to assess possible enduring effects of multiparity at least 3 months after last litter was weaned. MP females performed significantly better than NP females on the non-spatial memory task, object recognition, and the spatial memory task, object placement. Anxiety as measured on the elevated plus maze did not differ between groups. Monoaminergic activity and levels were measured in prefrontal cortex, CA1 hippocampus, CA3 hippocampus, and olfactory bulb (OB). NP and MP females differed in monoamine concentrations in the OB only, with MP females having significantly greater concentrations of dopamine and metabolite DOPAC, norepinephrine and metabolite MHPG, and the serotonin metabolite 5-HIAA, as compared to NP females. These results indicate a long-term change in OB neurochemistry as a result of multiparity. Brain-derived neurotrophic factor (BDNF) was also measured in hippocampus (CA1, CA3, dentate gyrus), and septum. MP females had higher BDNF levels in both CA1 and septum; as these regions are implicated in memory performance, elevated BDNF may underlie the observed memory task differences. Thus, MP females (experiencing multiple bouts of pregnancy, birth, and pup rearing during the first year of life) displayed enhanced memory task performance, but equal anxiety responses, as compared to NP females. These results are consistent with previous studies showing long-term changes in behavioral function in MP, as compared to NP, rats, and suggest that alterations in monoamines and a neurotrophin, BDNF, may contribute to the observed behavioral changes.

Keywords

reproductive experience; multiparity; recognition memory; monoamines; BDNF

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Rats with reproductive experience (pregnancy, birth and pup rearing) demonstrate persistent behavioral changes which can outlast the period of maternal care, the most well-known of which is a prolonged memory for and maternal response towards pups, months after their initial exposure (Bridges, 1975). In addition to the display of 'traditional' maternal behaviors, further behavioral alterations occur that may be essential for offspring care. Specifically, parous females (females who have given birth and lactated; see Kinsley et al., 1999) have a reduced fear response as compared to nulliparous females (NP: females with no mating or reproductive experience), which may be necessary to accept and care for formerly fear-inducing pups (Fleming and Luebke, 1981). Similarly, reproductive experience alters anxiety and the stress response. Parous females have significantly decreased c-fos mRNA expression in the hypothalamus, medial amygdala and lateral septum after restraint stress in comparison to NP females (da Costa et al., 1996; Wartella et al., 2003). Parous females also have decreased anxiety-like behavior as measured in the open field (Wartella et al., 2003) and on the elevated plus maze (Neumann, 2001; Lonstein, 2005). Furthermore, recent work indicates that multiparity continues to decrease anxiety as measured on the elevated plus maze up to 18 months after last weaning (Love et al., 2005), indicating effects on behavior throughout the female's lifespan.

When tested less than three months after last parturition, females with prior reproductive experience show enhanced spatial memory (Kinsley et al., 1999; Pawluski et al., 2006a) and foraging ability (Lambert et al., 2005) as compared to NP females, which may allow dams to spend less time and energy away from pups while searching for food. Interestingly, parous females (primiparous and multiparous) continue to outperform nulliparous females on a dry-land version of the Morris water maze at 6, 12, 18 and 24 months of age (Gatewood et al., 2005), and are significantly faster than NP females to find a baited food well at 13 months of age (Love et al., 2005), long past their last reproductive experience. However, one recent study indicated that, compared to NP females, primiparous but not multiparous females have significantly better spatial memory on the radial arm maze (Pawluski et al., 2006b). Thus, while parity reliably enhances spatial memory, whether multiple reproductive experiences further benefits memory warrants further investigation.

The mechanisms underlying the enduring effects of parity on anxiety and memory remain largely unexplored. Previous research has focused primarily on structural and synaptic changes in the hippocampus (Tomizawa et al., 2003; Kinsley et al., 2006), neurogenesis in the olfactory bulb (Shingo et al., 2003), and monoaminergic neurotransmitters in the medial preoptic area of the hypothalamus (Lonstein et al., 2003). In the current study, we have chosen to focus primarily on three regions associated with memory task performance: the hippocampus, prefrontal cortex, and olfactory bulb. Based upon abundant literature indicating a role in memory task performance, two neurochemical systems (monoaminergic neurotransmitters and brain-derived neurotrophic factor, BDNF) were examined in the above regions as possible means by which reproductive experience could affect memory task performance, likely through altered release or activity.

Specifically, memory task performance is affected by monoaminergic activity in the hippocampus (Lemon and Manahan-Vaughan, 2006; O'Carroll et al., 2006), prefrontal cortex (Marrs et al., 2005; Moore et al., 2005; Lapiz and Morilak, 2006), and olfactory bulb (Fleming and Rosenblatt, 1974; Pissonnier et al., 1985; Dickinson and Keverne, 1988; Levy et al., 1990; Calamandrei et al., 1992; Guan and Dluzen, 1994). If elevations in monoamines during pregnancy and lactation remain long-term in the aforementioned brain regions, then these neurochemicals may underlie the effects of parity on memory task performance.

BDNF is found throughout the brain and has many roles, including neuronal maintenance into adulthood (Allen and Dawbarn, 2006). In particular, hippocampal BDNF is of importance due

to its role in learning and memory (Alonso et al., 2002; Tyler et al., 2002), particularly spatial memory (Radecki et al., 2005), as well as the well-accepted cellular substrate of learning and memory, long-term potentiation (Ying et al., 2002; Pang and Lu, 2004). Cholinergic input from the septum seems to modulate spatial memory (Smith and Pang, 2005); these same neurons transport BDNF protein from hippocampus to septum (Sobreviela et al., 1996). Furthermore, BDNF protein levels are naturally elevated during proestrus and estrus (Scharfman et al., 2003), and changes in BDNF mRNA and protein have been reported in ovariectomized rats after acute (Gibbs, 1999) and chronic (Zhou et al., 2005) estradiol treatment (for review see Scharfman and MacLusky, 2006). If multiparity exerts long-term changes on hippocampal (or septal) BDNF protein, it is possible that BDNF may underlie any positive effects of parity on spatial memory.

In this study, age-matched nulliparous (NP: virgin) and multiparous (MP: 5–6 litters) female rats were compared on a non-spatial memory task, object recognition (OR), and a spatial memory task, object placement (OP). To date, these two tasks have not been used to examine memory changes due to reproductive experience, and the effects of parity as defined here (5–6 litters) have yet to be assessed in relation to memory ability. In addition, anxiety was assessed on the elevated plus maze (EPM) to determine any anxiolytic effect of multiparity. Monoaminergic levels and activity, and BDNF, were measured in a variety of brain regions as possible mechanisms underlying possible behavior differences. Results show that 1) multiparity is associated with better memory, but does not affect anxiety; 2) multiparity significantly alters monoaminergic concentration and activity in the OB; and 3) MP females have elevated BDNF protein levels in CA1 hippocampus and septum as compared to NP females.

Methods

Cohort 1

Subjects—Sixteen 11-month-old Fisher-344 rats (nulliparous: NP = 8; multiparous: MP = 8) were obtained from the NIA colony at Harlan, Inc (Indianapolis, IN) and double-housed under a 12:12 light:dark cycle (lights on at 0500 h) with water and food (Harlan Teklad Rodent Chow) available *ad libitum*. Upon arrival in our facility, MP females were approximately one month past last weaning of pups, and had at least 5 litters throughout the first year of life. There were no weight differences between the groups. Experiments began after a two-week acclimation period to their home cage, during which all subjects were handled daily by the experimenter. All testing occurred between 1000 and 1500 hours. All procedures used were approved by the IACUC at Hunter College of the City University of New York.

Following behavioral testing and prior to sacrifice at 13 months of age, one 13-month old NP female died and her blood and tissue were therefore unavailable for hormonal or neurochemical analyses. All other females were sacrificed by immediately after completion of all behavior tasks (see below) by decapitation following light anesthesia by carbon dioxide. Brains were removed and blocked just behind the prefrontal cortex (PFC; removed and frozen on dry ice) and just anterior to the cerebellum (discarded), then quickly frozen in dry ice and stored at −70°C to prevent degradation of the samples until brain neurochemical analysis (see below).

Object recognition and placement tasks—Rats were habituated to the object recognition (OR) and object placement (OP) tasks as described previously (Bisagno et al., 2002; Luine et al., 2003). Briefly, each task consisted of a sample trial (T1) and a recognition/retention trial (T2). Rats were given four days of exposure to the OR task with increasingly larger inter-trial delays (1 min, 10 min, 1 hour, 2 hour) between T1 and T2; delays of 2 and 4 hours were used for testing. During T1, two identical objects were placed at one end of an open field (70 × 70cm × 30cm high); the amount of time the rat explored both objects was recorded

for 3 min. During T2, at the end of the inter-trial delay, one object was replaced; exploration of the old and new object was recorded. Following habituation, OR was tested over two days, with a 2 hour inter-trial delay followed by a 4 hour inter-trial delay; these tests were used for data analysis

Rats were subsequently given four days of habituation to the OP task with increasingly larger inter-trial delays (10 min, 40 min, 1 hour, 2 hour) between T1 and T2; delays of 2 and 4 hours were used for testing. T1 was identical to the OR task; during T2, the same two objects were again presented, with one object moved to a new location. Following habituation to OP, testing occurred over two test days, with a 2 hour inter-trial delay followed by a 4 hour inter-trial delay; these tests were used for data analysis. Throughout habituation and testing on both tasks, novel objects were presented each day. Exploration of the objects was defined as any time in which the subject sniffed at, whisked at, or looked at the objects from no more than 2 cm away. Females were approximately 12 months old at completion of OR and OP tasks.

Elevated Plus Maze—Elevated plus maze (EPM) protocol was adapted from Pellow et al. (1985) and consisted of two open arms (50cm × 10cm) and two closed arms (50cm × 10 cm × 40cm high) elevated 40cm above the floor. The walls extended from a central neutral area (10 × 10cm) not counted in exploration or entries; subjects were placed in the neutral area facing one of the open arms and given five minutes to explore the maze. The number of entries into open and closed arms, and duration of entries into the open and the closed arms, were recorded. A total of three paws inside of an arm were used as criteria for entry. Time spent in the central area was not recorded. All subjects were tested in the EPM three weeks after completion of OR and OP tasks and sacrificed immediately following completion of the EPM task.

Radioimmunoassay—Serum hormones (testosterone and progesterone) were measured as described in Sharfman et al (2003, 2005) utilizing Coat-a-Count kits from Diagnostic Products Corporation (DPC: testosterone, catalog #TKTT1; progesterone, catalog #TKPG1), according to the kit instruction. Briefly, to each 50μl sample, 1ml of ^{125}I-testosterone or ^{125}I-progesterone was added, vortexed briefly, then incubated overnight at 4°C. The following day, the liquid was poured off and bound isotope was counted in a Wizard 1470 Automatic Gamma Counter (PerkinElmer Life Sciences, Wellesley, MA). Amount of testosterone and progesterone was calculated by comparison with the standard curve using log-logit transformation of the data. The minimum detectable concentrations of hormone, defined as being greater than the 95% upper confidence limit for the zero standard, were 10pg/ml for testosterone and 0.1 ng/ml for progesterone. The intra- and inter-assay coefficients of variation were below 10% for both assays.

Serum estradiol was measured using a #TKE21 kit (Coat-a-Count from DPC) with prior ether extraction (see Scharfman et al., 2003) to separate estradiol from the binding proteins present in rat, but not human, serum. Aliquots (100 μl) of serum were extracted with 2 × 1ml anhydrous ethyl ether from a freshly opened can of anesthetic ether; the extracts were transferred to 12 × 75 mm glass tubes and dried under a stream of air. Equal volumes of serum from the kit standards were extracted in parallel, to correct for procedural losses. The dry extracts from both samples and standards were redissolved by addition of 1ml of the kit I^{125} estradiol solution and vortexed thoroughly. The assay was then conducted as per kit instructions, and concentrations determined as in testosterone and progesterone assays. The minimum detectable concentration of estradiol was 2 pg/ml. The intra- and inter-assay coefficients of variation were below 10%.

Monoamine and metabolite analysis—Frozen brains were sliced in half sagitally. One hemisphere was sectioned into 300μm sections through the hippocampus using a cryostat at −4°C. The frozen PFC and hippocampal sections were sampled with a 500μm-diameter cannula

(4–6 tissue punches from each animal in the PFC, and 10–12 tissue punches from each animal in CA1 and CA3). Monoamines and metabolites were measured through high performance liquid chromatography (HPLC) with electrochemical detection (E.C.): dopamine (DA) and two metabolites 3,4-dihydroxy-phenylacetic acid (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG); serotonin (5HT) and metabolite 5-hydroxy indole acetic acid (5-HIAA). The procedure was as described previously (Bisagno, Ferguson & Luine, 2002). To each tissue sample, 60μl sodium acetate buffer (pH 5.0) with α-methyl-dopamine as an internal standard was added. The samples were frozen, thawed, the supernatant drawn off, and the pellet re-suspended in 100μl (PFC: 4–6 tissue punches) or 200μl (CA1/CA3: 10–12 tissue punches) of 2.0N NaOH for protein analysis using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA). The supernatant containing the monoamines and metabolites was injected into a Waters Associates chromatographic system (Waters 2690), consisting of an automated refrigerated injector, pump, C-18 reverse-phase column (Novapak three micron), and an ESA Coulochem III detector (-150mV to 0.50V potential). The mobile phase, described elsewhere (Luine and Hearns, 1990), contained 3% acetonitrile and peak sharpness was increased by the addition of 100% methanol (99.5% mobile: 0.5% methanol). Millennium software (Waters Associates) was used to run the chromatography system, in which concentrations of transmitters and metabolites were calculated by reference to standards using peak integration. Monoamine concentrations are expressed as pg/μg protein.

BDNF analysis—From the other half of each brain, the entire hippocampus and septum were dissected out and stored individually at −70°C until analysis of BDNF via enzyme-linked immunosorbent assay (ELISA). The tissue was weighed and placed into 1000μl of Lysis buffer (Promega). The solution was homogenized using a mechanical homogenizer from IKA (Eurostar power-control 6000). Following centrifugation for 30 minutes at 13,000rpm, the supernatant was removed (pellet was discarded). Seven serially-diluted standards were made from the BDNF standard in the Elisa kit (Chemicon, Intl #CYT306) ranging from 0–500 pg/ml, and 100μl of each was added to the microplate. For samples, 25μl of each sample was mixed with 75μl of diluent. Each standard and sample was measured in duplicate. After addition to the microplate, the samples were incubated overnight at 4°C.

On the second day, the wells were washed 4 times with wash buffer; 100μl of biotinylated mouse anti-BDNF antibody (1:1000) was added to each well and incubated for 2.5 hours at room temperature. Samples were washed with buffer 4 times; 100μl of streptavidin-HRP conjugate (1:1000) was added to each well and incubated for 1 hour at room temperature. Samples were washed 4 times; 100μl of TMB/E solution (at room temperature) was added to each well and incubated at room temperature for 15 minutes. 100μl of stop solution was then added to each well and the microplate was immediately placed into microplate reader (Bio-Tek EL312, interfaced with Dell Optiplex GS computer running KC Junior software) and measured at 450nm. Optical density units were converted to nanograms of BDNF using the generated standard curve. Concentrations are expressed as ng/g wet weight.

Statistical analysis—All data analysis was conducted using SPSS™ software (SPSS Inc, Chicago, Illinois). Data from the OR and OP tasks were analyzed as follows. Data from the sample trial (T1) of both tasks were analyzed with a two-way ANOVA (group × delay). Data from the recognition/retention trial (T2) of both tasks (exploration ratio: time with new object/time with old + time with new) were analyzed with a two-way ANOVA (group × delay). Data from the EPM were analyzed with a two-way ANOVA (group × entries) for number of entries into open arms and duration of entries into open arms.

Data from each RIA were analyzed with a t-test. Monoamines, metabolites and turnover ratios in each brain region (CA1, CA3, PFC) were analyzed with a two-way ANOVA (group ×

neurochemical). Monoamine analysis was carried out on data from seven MP and six NP females, as data from one animal in each group were statistical outliers (over 2 standard deviations from the mean) and therefore excluded. BDNF levels were analyzed separately in hippocampus and septum using a two-way ANOVA (group × region). Post hoc analysis of monoamines and BDNF used independent-samples t-tests. For all statistical tests, $p < 0.05$ for significance.

Cohort 2

Subjects—A second cohort of fourteen 12-month-old Sprague-Dawley rats (NP = 6; MP = 8) were obtained after use at Helen Hayes Hospital (West Haverstraw, NY). The MP females had been used solely for breeding for 6 months prior to this study (4 with four litters, 2 with 3 litters, and 2 with two litters), and the NP females were housed in pairs and had no history of behavioral or reproductive use. These subjects were used to measure monoamine and metabolite concentration in the olfactory bulb (OB), as well as BDNF concentration in subregions of the hippocampus.

All subjects were sacrificed by decapitation following light anesthesia by carbon dioxide. Brains were immediately removed, placed into dry ice, and stored at −70°C to prevent degradation of the samples. Each brain was then divided into 6–7 thick sections based upon anatomical markings (sections made at olfactory bulb (OB), prefrontal cortex, anterior to optic chiasm, posterior to optic chiasm, just anterior to the hypothalamus, and just posterior to the hypothalamus; see Luine et al., 1974).

Monoamine analysis—Brains sections containing the OB were sampled with a 500μm-diameter cannula (4–6 tissue punches were obtained from each animal). Tissue punches were taken under a dissecting microscope, on a microscope stage maintained at −4°C. Monoamine and metabolite concentrations were measured in the same manner as described for Cohort 1.

BDNF analysis—A 500μm-diameter cannula was used to sample tissue from three subregions of the hippocampal formation: CA1, CA3, and dentate gyrus (DG). Tissue punches were taken under a dissecting microscope, on a microscope stage maintained at −4°C. BDNF levels using the ELISA kit and procedure described above for Cohort 1 was used, with minor modifications. As the tissue punches contained less tissue than the entire hippocampus, it was homogenized in 250μl of Lysis buffer (Promega) to prevent dilution and the full 100μl of sample specified in the kit was added to each well. As tissue weight could not be obtained, total protein levels were measured by Bradford assay (see da Silva and Arruda, 2006). BDNF concentration is expressed as ng/g protein.

Statistical analysis—Monoamine concentrations in the OB were examined using a two-way ANOVA (group × neurochemical) for monoamines, metabolites, and turnover ratios. BDNF protein levels were analyzed using a two-way ANOVA (group × region). For all statistical tests, post-hoc analysis was carried out using independent-samples t-tests, and $p < 0.05$ for significance.

Results

Object Recognition

The OR task was administered at 2- and 4-hour inter-trial delays. In the sample trials (T1), no significant differences were found between groups (Figure 1A) indicating equal overall exploration between NP and MP females. In the recognition trial (T2), there was a significant group effect ($F_{1,28} = 6.80$, $p < 0.02$), as well as a significant group × delay interaction ($F_{1,28} = 4.08$, $p < 0.05$). MP females had significantly higher exploration ratios than did NP females

(approximately 0.85 and 0.37, respectively) at the 2 hour ($p < 0.001$), but not the 4 hour delay (Figure 1B). At the 4 hour inter-trial delay, NP and MP females had similar exploration ratios, (approximately 0.54 and 0.60, respectively) indicating similar exploration of both objects by both groups.

Object Placement

As in OR, the OP task was administered at 2- and 4- hour inter-trial delays. In the sample trial (T1), there was a significant effect of delay ($F_{1,28} = 9.66$, $p < 0.01$). Both groups spent significantly greater time exploring the objects during the sample trial prior to the 4 hour delay than prior to the 2 hour delay (Figure 2A). However, there was no significant group effect ($F_{1,28} = 0.36$, $p > 0.10$), or group × delay interaction ($F_{1,28} = 0.16$, $p > 0.10$), indicating equal exploration between groups at each delay.

In the recognition trial (T2), there was a highly significant group effect ($F_{1,28} = 30.69$, $p < 0.001$), but no significant effects of delay, or group × delay interaction (Figure 2B). Thus, at both inter-trial delays, MP females had significantly higher exploration ratios (2 hour: 0.79, 4 hour: 0.83) than did NP females (2 hour: 0.23, 4 hour: 0.35).

Elevated Plus Maze

A two-way ANOVA (group × entries) revealed no significant differences in entries into the open arms (NP: 4.13 ± 0.77; MP: 5.38 ± 0.84) or duration of entries into open arms (NP: 69.94 ± 18.64; MP: 77.62 ± 15.56) between NP and MP females.

Serum gonadal hormones

No significant differences were present between NP and MP females in serum estradiol, testosterone, or progesterone levels (Table 1).

Brain monoamines and metabolites

Monoamine and metabolite concentrations (DA and metabolites DOPAC and HVA, NE and metabolite MHPG, 5HT and metabolite 5-HIAA) were measured in three brain regions: CA1, CA3 hippocampus, and PFC (Table 2). In each brain region, data was analyzed by a two-way ANOVA (group × neurochemical). There were no effects of group, or group × neurochemical interactions in PFC ($F_{1,11} = 1.03$, $p > 0.50$), in CA1 ($F_{1,11} = 12.30$, $p > 0.20$), or in CA3 ($F_{1,11} = 1.07$, $p > 0.50$). Concentrations of monoamines, metabolites, and turnover ratios (data not shown) did not significantly differ between NP and MP females in these brain regions.

A second cohort of NP and MP females (without behavior experience; see Method) was used to measure monoamines, metabolites and turnover ratios in the olfactory bulb (OB) (Figure 3). A two-way ANOVA (group × neurochemical) revealed a significant overall effect of group ($F_{1,12} = 58.62$, $p < 0.05$). Compared to NP females, MP females had significantly higher levels of DA ($p < 0.01$) and the metabolite DOPAC ($p < 0.001$; Figure 3A); significantly higher levels of NE ($p < 0.001$) and metabolite MHPG ($p < 0.001$; Figure 3C); and the 5HT metabolite 5-HIAA ($p < 0.01$; Figure 3E).

Turnover ratios (metabolite/monoamine) were calculated as a measure of monoaminergic activity. Compared to NP females, MP females had significantly higher turnover ratios of DOPAC/DA ($p < 0.01$; Figure 3B) and 5-HIAA/5HT ($p < 0.05$; Figure 3F). In contrast, as compared to NP females, MP females had a significantly lower turnover ratio of HVA/DA ($p < 0.001$; Figure 3B) and did not differ in turnover ratio of MHPG/NE (Figure 3D). Combined, results indicate that parity significantly enhanced both concentrations and activities in the DA, NE and 5HT monoaminergic systems in the OB, with different patterns of activity for the DA metabolites DOPAC and HVA.

Brain BDNF

Concentrations of BDNF in the hippocampus and septum of NP and MP females are shown in Figure 4. Two-way ANOVA (group × region) revealed a significant effect of group ($F_{1,26}$ = 4.81, $p < 0.05$), and a significant group × region interaction ($F_{1,26}$ = 4.06, $p < 0.05$). BDNF concentration did not significantly differ in the hippocampus (Figure 4A), but MP females had significantly greater BDNF protein in the septum than NP females ($p < 0.05$; Figure 4A). Thus, parity enhanced BDNF concentration in the septum of MP females, but not in the hippocampus.

A second cohort of NP and MP females (without undergoing behavior testing; see Method) was used to measure BDNF concentration in three subregions of the hippocampus (CA1, CA3, DG). A two-way ANOVA (group × region) revealed no significant effect of group ($F_{1,12}$ = 1.73, $p > .05$). However, there was a significant group × region interaction ($F_{1,12}$ = 4.88, $p < 0.05$). BDNF concentration was significantly higher in the CA1 subfield of MP as compared to NP females ($p < 0.05$), but did not differ in either CA3 or DG (Figure 6B).

Discussion

The current study compared behaviors and neurochemicals in multiparous (MP) females, i.e. females having experienced 5–6 pregnancies, births, and lactation periods, with age-matched nulliparous (NP) females, i.e. virgin females with no mating or reproductive experience. Performance on tasks assessing memory, but not anxiety, was different. In addition, levels of monoamines and BDNF were different in some, but not all, brain areas examined.

Multiparity significantly enhanced memory performance

The current study presents novel findings that performance on a non-spatial task, OR, was better in MP as compared to NP females. At a 2 hour inter-trial delay, MP females spent over 80% of their time exploring the new object, whereas NP females spent approximately 40% of the time exploring the new object. Thus, NP females explored the new object at levels similar to chance, whereas MP females explored the new object significantly more than the old object, indicating a preference for the novel object. However, at a 4 hour inter-trial delay, NP and MP females did not significantly differ in performance; both groups explored the new object at levels similar to chance (approximately 50% of the time). Thus, multiparity was sufficient to enhance recognition memory at a short inter-trial delay (2 hour), but when the cognitive load was increased (i.e. inter-trial delay of 4 hours), effects of parity were no longer evident. These results are the first to report an enhancing effect of multiparity on non-spatial memory.

On the spatial memory task, OP, MP females outperformed NP females, spending approximately 80% of the time exploring the new object location in the recognition trial at both 2 and 4 hour inter-trial delays, while NP females spent less than 35% of the time exploring the new object location at both delays. Thus, NP females explored the new object location at levels similar to chance, whereas MP females explored the new object location significantly more than the old object, indicating a preference for the novel object location. These results are consistent with previous studies in which MP females had enhanced spatial memory on the radial arm maze while young (Kinsley et al., 1999; Pawluski et al., 2006a) and on the dry land maze throughout the lifespan (Gatewood et al., 2005).

Comparison of OP and OR results suggests that spatial memory may be more sensitive to the effects of multiparity than non-spatial memory. In both tasks, the time spent exploring objects during the sample trial was not significantly different between the two groups. This result suggests that performance differences on the recognition/retention trials (T2) between NP and MP females was not due to differences in exploratory drive, but may involve differences in memory processing.

Multiparity did not significantly alter anxiety

A significant increase in number and duration of entries into the open arm of the EPM is a reliable indicator of decreased anxiety (Pellow et al., 1985; Lonstein, 2005). In the current study, NP and MP females did not significantly differ in either measure of anxiety, indicating that anxiety-like behavior was not affected by previous reproductive experience. Previous work utilizing the EPM has indicated that anxiety is decreased during the post-partum period, when mothers are allowed to suckle their pups (Lonstein, 2005), and that reproductive experience earlier in life (less than 6 months old) decreases anxiety on the EPM at 10 and 14 months of age (Love et al., 2005). However, a recent study has indicated that on the EPM prior reproductive experience may be anxiogenic in middle-aged (10–11 month old), but not in young (3–4 month old) females (Byrnes and Bridges, 2006). The current findings indicate that multiparity is neither anxiolytic nor anxiogenic in middle aged females; as such, further exploration into the relationship between parity and anxiety, particularly in middle age, is required.

Multiparity did not affect serum hormone levels

NP and MP females did not significantly differ in measured levels of serum estradiol, testosterone, or progesterone, hormones that can affect memory and anxiety (Dohanich, 2002). While only examined on day of sacrifice, vaginal cytology indicated that the majority of females in both groups were in an estrus state, as evidenced by the presence of primarily cornified epithelial cells (data not shown). In Fisher rats, the onset of irregular estrous cycles begins around 12 months of age (Markowska, 1999), and is characterized by a prolonged cornified cell phase between 11–14 months of age (Sone et al., 2007). As estradiol and progesterone levels did not differ, and vaginal cytology appeared similar across both groups, prior reproductive experience did not appear to influence reproductive aging of the females. It is unlikely that performance differences between MP and NP females on the OR and OP tasks were due to differences in circulating gonadal hormone levels at time of testing.

Multiparity significantly altered monoaminergic function in olfactory bulb

Since monoamines are known to contribute to memory function (see Luine et al., 1998), we measured monoamine and metabolite concentrations in CA1, CA3 hippocampus and PFC. No significant differences in monoamine or metabolite levels, or in turnover ratios, were found between NP and MP females in PFC or hippocampus, indicating no effect of mulitparity on dopamine, norepinephrine or serotonin systems in these brain regions. In contrast, multiparity significantly affected monoamine systems in the OB; this study is the first to show large and enduring changes due to multiparity in this region.

Specifically, MP females had 30–75% higher levels of DA, DOPAC, NE, MHPG, and 5-HIAA than NP females. Additionally, turnover ratios of DOPAC/DA and 5-HIAA/5HT were elevated by 17% and 34%, respectively. In contrast, levels of the DA metabolite HVA did not significantly differ between NP and MP females, yet MP females had turnover ratios of HVA/DA that were 60% lower than in NP females. Combined, these results indicate greater intraneuronal synthesis of DA and metabolism to DOPAC, with less release into the synapse, corresponding with previous work in which parity increased DA and DOPAC levels in forebrain regions (Byrnes et al., 2001). Overall, the results indicate that while DA activity is decreased in the OB of MP females, synthesis and metabolism to DOPAC are elevated. Additionally, both levels and activity (as measured by conversion to MHPG) of NE are increased in the OB of MP females, as is activity of 5HT (as measured by conversion to 5-HIAA).

Previous research has indicated that as male and female rats age, levels of DA, NE and 5HT decrease throughout the brain: young rats (3–6 months old), as compared to aging rats (15

months or older), display higher monoamine levels in striatum, entorhinal cortex, hippocampus, frontal cortex (Luine et al., 1990; Tanila et al., 1994; Miguez et al., 1999), and cerebral cortex (Lee et al., 2001). By 15 months of age, significant decreases in the monoamine metabolites DOPAC, MHPG and 5-HIAA are observed in the OB of male rats (Dluzen, 1996) with corresponding decreases in olfactory-based memory (Guan and Dluzen, 1994). Thus, age-related activity changes in monoaminergic systems of the OB affect olfactory ability. Multiparity may maintain monoamine concentrations that ordinarily decrease with age, as higher concentrations of DA, NE, and metabolites DOPAC, MHPG and 5-HIAA were found in the OB of MP as compared to NP females.

Elevated monoamines may aid in offspring recognition, which is critically dependent upon the OB (Fleming and Rosenblatt, 1974; Brennan and Keverne, 1997), particularly NE projections throughout (Pissonier et al., 1985; Dickinson and Keverne, 1988; Levy et al., 1990; Calamandrei et al., 1992; Calamandrei and Keverne, 1994). Exposure to an odor-rich environment (such as a litter of pups) enhances olfactory memory and OB neurogenesis (Rochefort et al., 2002), which is also increased during pregnancy when prolactin increases (Shingo et al., 2003). Additionally, oxytocin, which is elevated during lactation, aids in social memory of conspecifics by male rats through actions in the NE system in the olfactory bulb (Dluzen et al., 1998; Dluzen et al., 2000). Little to no research has examined olfactory bulb makeup in relation to olfactory-based memory in females; what information exists focuses on the maternal period (i.e. Calamandrei et al., 1992; Calamandrei and Keverne, 1994). This study is the first to indicate a role for olfactory bulb monoamines in performance on non-social memory tasks in females with prior reproductive experience. It is also notable that changes in OB monoamines are so long-lasting after parturition, indicating a relatively permanent change in OB makeup.

It is important to note that differences in monoamines between NP and MP females were not found in Cohort 1 animals with behavioral experience, but only in Cohort 2 animals without behavior experience. However, as sampling was done in different brain regions between the two cohorts (hippocampus and prefrontal cortex for Cohort 1; olfactory bulb in Cohort 2), it is reasonable that these differences are due to parity, and not behavior experience.

Multiparity significantly elevated BDNF concentration in CA1 hippocampus and septum

BDNF protein levels did not significantly differ in whole hippocampus of NP and MP females. However, BDNF was 51% higher in the CA1 hippocampal subregion of MP females as compared to NP females. No significant differences in BDNF between the two groups were found in CA3 or DG. Expression of BDNF protein in CA1 is typically much lower than in CA3 or DG, regardless of gender or serum estradiol levels, but this pattern of expression occurs when an antibody is used that preferentially identifies BDNF in nerve terminals (Conner et al., 1997; Yan et al., 1997; Scharfman et al., 2003). The ELISA kit used in the current study depended on an antibody that may preferentially localize BDNF protein in pyramidal cell bodies and dendrites (see Tongiorgi et al., 2006). Using this antibody, there were significant effects of multiparity on BDNF expression in CA1 hippocampus.

BDNF levels were also 45% higher in the septum of MP females relative to NP females, indicating an increase in BDNF expression throughout the septum. The medial septum contains cholinergic neurons responsible for retrograde transport of BDNF protein from hippocampus to septum (Sobreviela et al., 1996). Treatment with estradiol and progesterone decreases hippocampal BDNF protein, but increases hippocampal BDNF mRNA (Gibbs, 1999), suggesting either an increase in BDNF protein degradation or transport away from hippocampus (Sohrabji et al., 1995), possibly through septal cholinergic neurons. Repeated exposure to estrogen during pregnancies could have increased retrograde transport of hippocampal BDNF protein. However, there are limitations to measuring protein levels alone,

specifically that amount of synthesis and/or release of BDNF cannot be measured. To fully understand the effect of parity on BDNF in the hippocampus and septum, future studies should examine BDNF mRNA in these regions.

Additionally, it is possible that, as with the monoamine results, differences in hippocampal BDNF may have been due to the lack of behavioral experience in Cohort 2 animals, and not simply based upon the subregions sampled. However, the OR and OP tasks do not use a training paradigm, and as such traditional 'learning' does not occur (Ennaceur et al., 2005), which should eliminate possible confounds of behavioral experience between the two cohorts. As such, we believe that the differences in BDNF found between NP and MP females in the CA1 are in greater part due to parity, and not behavior experience.

Alternatively, MP females may have higher BDNF than NP females due to the physical aspect of rearing pups. New dams display a variety of physical behaviors such as nest building, crouching over pups, and pup retrieval, that were not displayed prior to pregnancy and parturition (Rosenblatt et al., 1988). Exercise in female rats (several days of voluntary wheel-running) significantly increases BDNF mRNA and protein in the hippocampus, particularly in the CA3 and DG (Berchtold et al., 2001; Berchtold et al., 2002; Cotman and Berchtold, 2002). Additionally, exercise and the subsequent increase in BDNF protein act together to enhance spatial memory as measured on the Morris water maze (Vaynman et al., 2004). If maternal behaviors, including nest building and retrieval of pups, increase physical activity in new dams in a manner similar to voluntary wheel running, then the recurring display of these behaviors with multiparity may increase BDNF levels in the hippocampus to a point that is beneficial to spatial memory.

We suggest here that BDNF in CA1 pyramidal cells and the septum may underlie memory task performance. This hypothesis is supported by the evidence to date that BDNF is important for LTP in CA1 hippocampus (Ying et al., 2002; Pang and Lu, 2004), that BDNF plays an important role in learning and memory (Alonso et al., 2002; Tyler et al., 2002; Alonso et al., 2005), and specifically is involved in spatial memory (Linnarsson et al., 1997; Mu et al., 1999; Mizuno et al., 2000; Radecki et al., 2005). This hypothesis is also consistent with a role for both CA1 and septum in memory performance. Lesions to the CA1 significantly impair performance on OR and OP tasks (Broadbent et al., 2004; Gulinello et al., 2006); OP in particular is dependent on an intact hippocampus and/or fornix (Ennaceur and Aggleton, 1994; Ennaceur et al., 1997; Mumby et al., 2002; Broadbent et al., 2004). Similarly, lesions to the dorsal hippocampus (containing CA1 and septal regions) impair performance on the Morris water maze to the same extent as lesions of whole hippocampus (Clark et al., 2005). Neurons in the medial and lateral septum fire with hippocampal neurons, and aid in processing spatial information (Zhou et al., 1999); lesions to the septum interfere with both hippocampal firing and spatial working memory (Leutgeb and Mizumori, 1999). The CA1 and septum are thus particularly important for spatial memory task performance, and to a lesser extent non-spatial memory (Ennaceur and Aggleton, 1994; Ennaceur et al., 1997; Broadbent et al., 2004). Current findings of elevated BDNF in the CA1 of MP females as compared to NP females likely contributed to enhanced performance on the object recognition and placement tasks.

In conclusion, these results show enduring changes in brain function and chemistry in rats experiencing several previous pregnancies. Furthermore, these changes persist many months after last reproductive experience, indicating possible semi-permanent or permanent changes in cognitive function and neurochemistry. More research into the factors contributing to these changes, particularly the relationship between multiparity and hippocampal BDNF, may lead to a greater understanding of amelioration of certain kinds of neurodegenerative and age-related losses in brain function.

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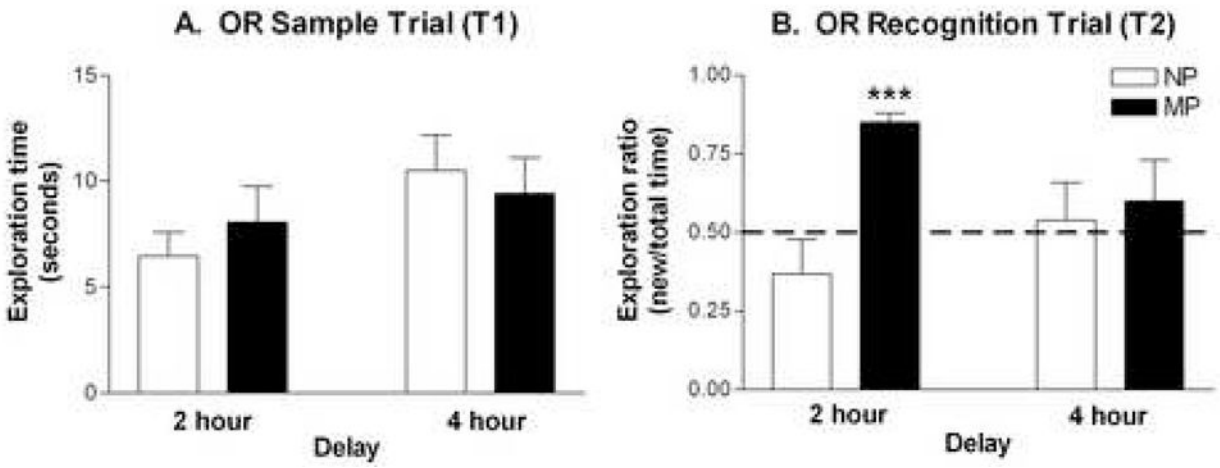

Figure 1. Effect of parity on object recognition

(A) Mean exploration times (± SEM) during the sample trial (T1) are shown for NP (□; n = 8) and MP (■; n = 8) females. No significant differences by two-way ANOVA. **(B)** Mean exploration ratios (± SEM) during the recognition trial (T2) are shown for NP and MP females. The exploration ratio is calculated as [time with new object/time with old object + time with new object]. Dashed line at 0.50 indicates chance performance on the task (equal time exploring old and new objects). ***$p < 0.001$.

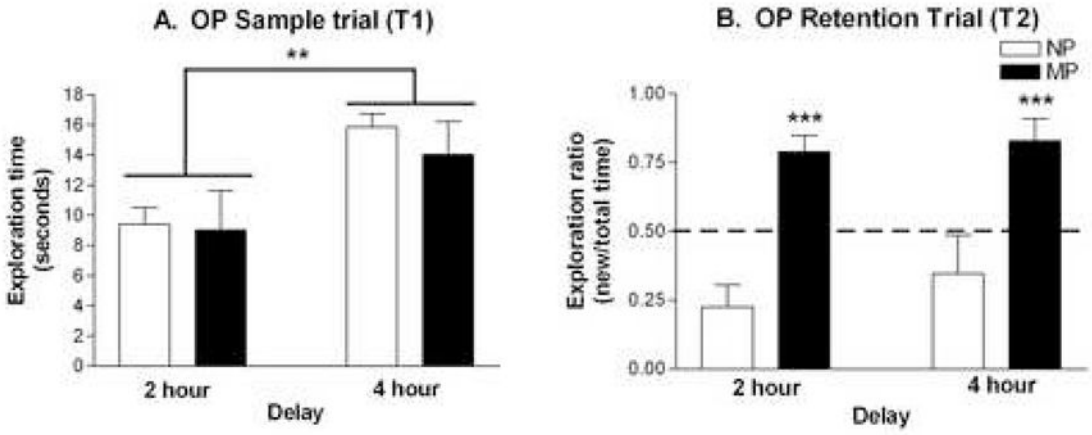

Figure 2. Effect of parity on object placement

(A) Mean exploration times (± SEM) during the sample trial (T1) is shown for NP (□; n = 8) and MP (■; n = 8) females. Data were analyzed by two-way ANOVA (group × delay). **(B)** Mean exploration ratios (± SEM) during the recognition trial (T2) is shown for NP and MP females. The exploration ratio is calculated as [time with new object/time with old object + time with new object]. Dashed line at 0.50 indicates chance performance on the task (equal time exploring old and new objects). $^{**}p < 0.01$; $^{***}p < 0.001$.

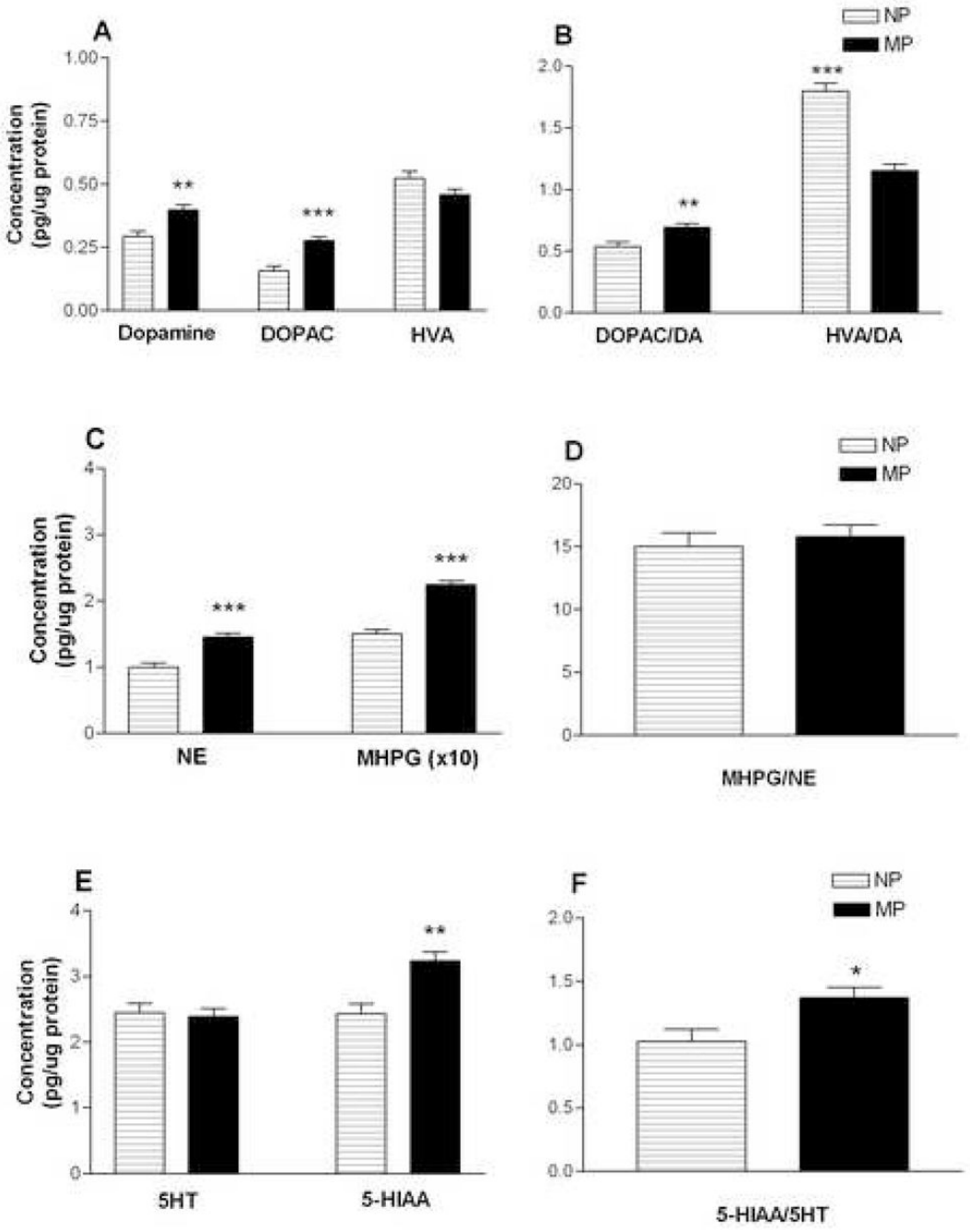

Figure 3. Effect of parity on monoamines, metabolites, and turnover ratios in the OB

Mean concentration (± SEM) of monoamines, metabolites and turnover ratios for NP (□; n = 6) and MP (■; n = 8) females. Concentrations are pg/μg total protein. DA, dopamine; DOPAC, 3,4-dihydroxy-phenylacetic acid; HVA, homovanillic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5HT, serotonin; 5-HIAA, 5-hydroxy indole acetic acid. Data were analyzed by two-way ANOVA (group × neurochemical). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

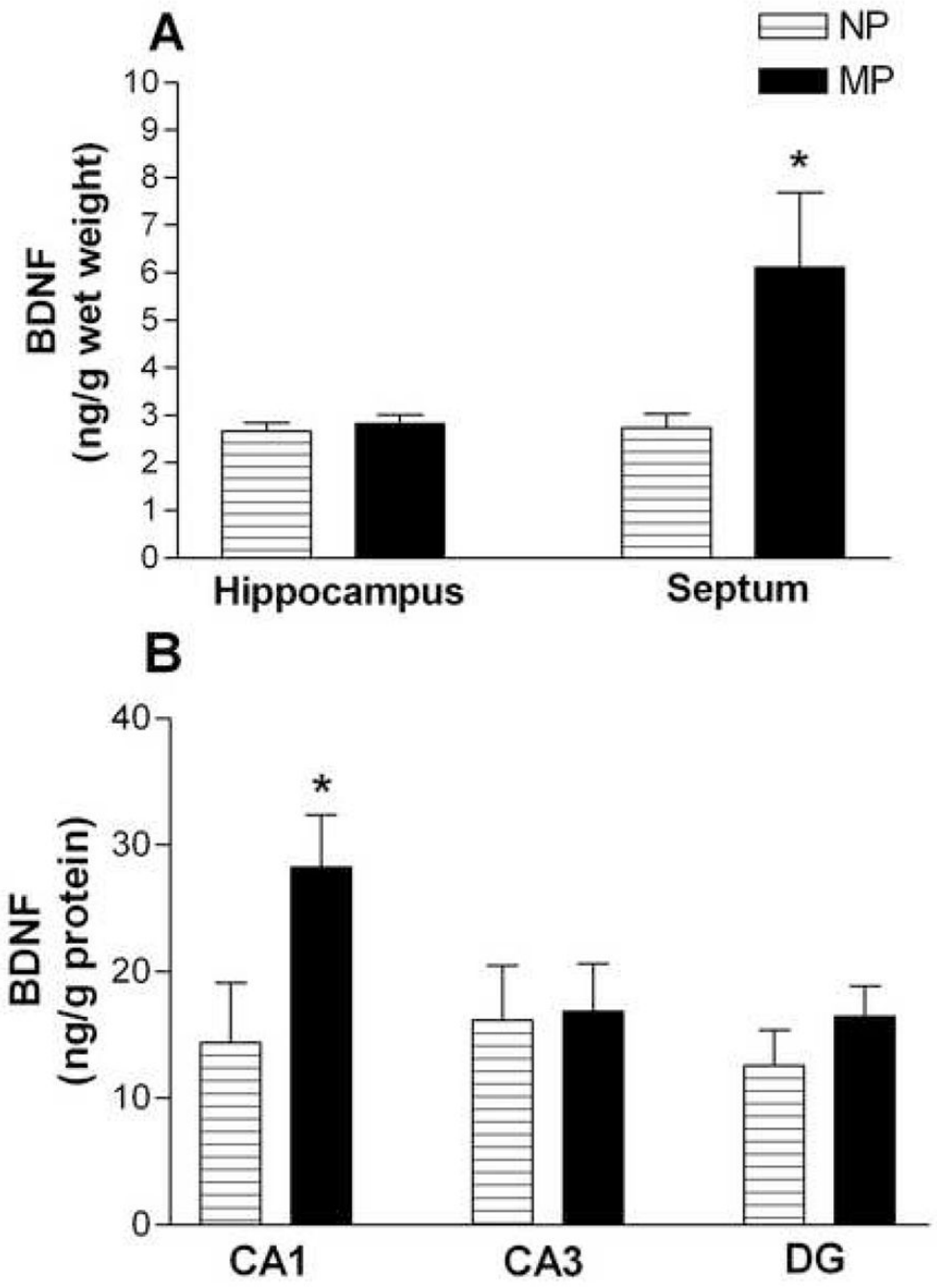

Figure 4. Effect of parity on BDNF expression in hippocampus and septum

(A) *Cohort 1.* Mean BDNF concentration (± SEM) in whole hippocampus (*left*) and septum (*right*) for NP (□; n = 7) and MP (■; n = 8). Concentrations given are in ng/g wet weight. **(B)** *Cohort 2.* Mean BDNF concentration (± SEM) in three hippocampal subregions: CA1 (*left*), CA3 (*middle*), and dentate gyrus (DG; *right*) of NP (□; n = 6) and MP (■; n = 8) females. Concentrations given are ng/g protein. Data from each cohort were analyzed by two-way ANOVA (group × region). * $p < 0.05$.

Table 1

Serum gonadal hormone levels in nulliparous and multiparous females

Entries are mean serum hormone levels ± SEM for NP (n = 7) and MP (n = 8). Data were analyzed by t-test for each hormone.

Group	Estradiol (pg/ml)	Testosterone (pg/ml)	Progesterone (ng/ml)
Nulliparous (NP)	24.14 ± 4.88	13.29 ± 3.29	13.71 ± 1.73
Multiparous (NP)	21.38 ± 4.50	34.13 ± 17.02	9.75 ± 2.45

Table 2

Effect of parity on monoamines and metabolites in three brain regions

Entries are mean concentration ± SEM for NP (n = 6) and MP (n = 7) females. Concentrations given are in pg/μg total protein. DA, dopamine; DOPAC, 3,4-dihydroxy-phenylacetic acid; HVA, homovanillic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5HT, serotonin; 5-HIAA, 5-hydroxy indole acetic acid. Data were analyzed by two-way ANOVA (group × neurochemical) with no significant differences in any brain region (PFC, CA1, CA3).

NT	Group	PFC	CA1	CA3
DA	NP	1.32 ± 0.13	0.64 ± 0.54	0.96 ± 0.27
	MP	1.10 ± 0.15	0.35 ± 0.13	0.87 ± 0.10
DOPAC	NP	0.82 ± 0.10	0.51 ± 0.20	0.92 ± 0.27
	MP	0.77 ± 0.13	0.26 ± 0.06	0.75 ± 0.09
HVA	NP	0.72 ± 0.09	1.36 ± 0.47	1.40 ± 0.44
	MP	0.71 ± 0.12	0.72 ± 0.18	1.29 ± 0.21
NE	NP	3.42 ± 0.46	2.54 ± 0.52	6.09 ± 0.94
	MP	3.54 ± 0.39	2.84 ± 0.34	6.10 ± 0.54
MHPG	NP	4.39 ± 0.90	15.35 ± 1.76	6.55 ± 0.98
	MP	3.00 ± 0.38	17.05 ± 4.00	4.97 ± 0.83
5HT	NP	5.44 ± 0.71	2.77 ± 0.88	3.54 ± 0.33
	MP	4.87 ± 0.63	2.64 ± 0.43	3.14 ± 0.24
5-HIAA	NP	5.50 ± 0.83	4.21 ± 0.75	4.90 ± 0.33
	MP	6.32 ± 1.40	4.54 ± 0.69	4.92 ± 0.28