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The Effects of Parity and Maternal Behavior on Gene Expression in the Medial Preoptic Area and the Medial Amygdala in Postpartum and Virgin Female Rats: A Microarray Study

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To determine the pattern of gene expression in brains associated with mothering during the postpartum period, in the present study we assessed gene expression through microarrays in four groups of female rats: two groups of new mothers that were experiencing the hormonal and neurochemical changes associated with pregnancy and parturition, and two groups of virgin females that were not. Within each of these parity groups we assessed one group of animals that was exposed to and responded to pups and engaged in maternal behavior, and one group left without any exposure to pups and therefore had no maternal experience. We explored the pattern of expression of genes related to the hormones, neurotransmitters, and modulatory neuropeptides associated with maternal behavior within the medial preoptic area (MPOA) and the medial amygdala (MeA) in the rat. Within the MPOA there were significant main effects of pup exposure for the dopamine-related genes (DRD4 and dopamine transporter, DAT), the glucocorticoid-related gene (CYPX1B1a), the opioid receptor μ-1 gene (OPRM1) and the gamma-aminobutyric acid (GABA) receptor gene (GABAbRid). OPRM1 and the serotonin-related gene that regulates biosynthesis of serotonin (5HTR2A) showed a main effect of parity. For both sets of analyses, higher gene expression was associated with pup exposure and parity. Genes expressed in the MeA tended to reside in the glucocorticoid family. The microarrays were able to identify, on a transcriptional level, a list of candidate genes involved in maternal behavior and the factors that surround it.

Keywords: Limbic, dopamine, hypothalamus, RNA, mother

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Many hormones and neurotransmitters are implicated in the onset and expression of maternal behavior, acting at multiple sites in the brain and affecting a variety of behavioral systems (Numan, Fleming, & Levy, 2006). The levels of these hormones and neurochemicals result from genes that are expressed in the brain and organ systems during parturition and in the early postpartum period. We focus on expression of candidate genes known to play a role in mothering, either through the new mother's endocrine state or in response to pup stimulation.

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The onset of mothering behavior (retrieval, licking, nursing, and nest-building) with the birth of the young is facilitated by late gestational increases in the ovarian steroid estradiol, and against a backdrop of initially high and then declining levels of progesterone (Bridges, 1984; Numan et al., 2006). The peptide hormones, prolactin and oxytocin, also rise at parturition and further facilitate the onset of maternal behavior by acting on the medial preoptic area (MPOA), which is considered, the "final common path" of the neural circuitry regulating the expression of maternal behavior (summarized in Numan et al., 2006). Their effects in the behaving animal are largely excitatory. Although not necessary for the onset or expression of maternal behavior in the postpartum animal, corticosterone also has modulating effects on maternal behavior (Graham, Rees, Steiner, & Fleming, 2006; Rees, Panesar, Steiner, & Fleming, 2004; Rees, Steiner, & Fleming, 2006a; Wong, Brummelte, & Galea, 2011). Also, in the virgin animal, who is initially avoidant with pups, the effects of corticosterone tend to be inhibitory (Numan, 1988; Rosenblatt, 1967; Rees et al., 2004; Rees, Steiner, & Fleming, 2006b).

The priming of the brain by a set of hormones is generally accomplished by the induction of receptors in the brain that bind and thereby enable the action of a subsequent set of hormones (see Numan et al., 2006; Numan & Insel, 2003). Moreover, variations in maternal licking in the postpartum animal are related to variations in receptors such as oxytocin (OTR; Francis, Champagne, & Meaney, 2000) and estrogen (ER; Champagne, Weaver, Diorio, Sharma, & Meaney, 2003).

The brain systems and their neurotransmitters most affected by these hormones are well-mapped. The neurotransmitter dopamine (DA) figures prominently in the pathways on which the hormones act and regulate the expression of maternal behavior. These include the MPOA, a structure that contains heavy densities of receptors for estrogen, progesterone, prolactin, and oxytocin as well as for the neurotransmitter, DA. This region also receives extensive DA projections from the hypothalamus and the ventral tegmental region (Numan & Stoltzenberg, 2009; Numan, Fleming, & Levy, 2006) and shows dendritic plasticity in response to parturition and maternal experience (Shams et al., 2012). The DA system and its receptors are strongly implicated in both the initiation of maternal retrieval and memory (Numan & Stolzenberg, 2009; Parada, King, Li, & Fleming, 2008; see also Afonso, Grella, Chatterjee, & Fleming, 2008) and in associated motivational, reward, and attentional systems (Kringelbach & Berridge, 2010; Pezze, Dalley, & Robbins, 2007; Afonso, Shams, Jin, & Fleming, 2013). The DA receptors of relevance include DRD1, DRD2, DRD3, and DRD4 (Numan & Stolzenberg, 2009; Li et al., 2004; Keer & Stern, 1999; Champagne et al., 2004; Parada et al., 2008). The role of the serotonin (5HT) system in mothering has been less well-studied, although its effects are assumed to be largely excitatory to the new mother rat (Barofsky, Taylor, Tizabi, Kumar, & Jones-Quartey, 1983; Zhao & Li, 2010; Johns et al., 2005; Ferreira, Picazo, Uriarte, Pereira, & Fernandez-Guasti, 2000).

Other neuromodulator receptor systems and cells that are located within the MPOA likely exert their effects by enhancing or reducing the effects of DA and 5HT. The functionality of these neuromodulators in the mothering context is well established, with GABA (Arrati, Carmona, Dominguez, Beyer, & Rosenblatt, 2006; Byrnes, Lee, & Bridges, 2007) and opioids exerting an inhibitory effect (Sobor et al., 2010; Miranda-Paiva, Nasello, Yin, & Felicio, 2001; Mann, Kinsley, & Bridges, 1991), as well as oxytocin (Shahrokh, Zhang, Diorio, Gratton, & Meaney, 2010; Pedersen, Caldwell, Petersen, Walker, & Mason, 1992), glutamate (Geisler, Derst, Veh, & Zham., 2007; Numan, 2006), and nitric oxide synthase (NOS; Popeski & Woodside, 2004).

Many of these peptides and their receptors also reside in the amygdala and are known to be implicated in stress and emotion regulation, systems also important for mothering. These include the peptides GABA, glutamate, and oxytocin (Bosch, Meddle, Beiderbeck, Douglas, & Neumann, 2005; Leng, Meddle, & Douglas, 2008; Theodosis et al., 2006; Lin, Mao, Su, & Gean, 2010). The amygdala is a limbic brain region known to regulate emotion and exerts an inhibitory effect on the MPOA in terms of mothering (Numan et al., 2006). Virgins with amygdala lesions are released from that inhibition and express maternal behavior toward foster pups much more rapidly than virgins with intact amygdalas (Numan et al., 2006).

Most research on the physiology and neurobiology of mothering has involved the above-mentioned hormones and neurotransmitters, but usually has focused on one, or at most, two of these at a time (Numan et al., 2006). Although these studies have been essential to our understanding of the neurochemistry of maternal behavior, with the advent of microarray technology, it is now possible to study thousands of genes at the same time, and even study differential expression within different brain regions concurrently. This has the potential to more accurately reflect the complexity of the underlying physiological changes when a new mother becomes maternal and interacts with her young (see Gammie et al., 2005).

In the present study, we assessed the pattern of gene expression in four groups of female rats: two groups of new mothers, who experienced the hormonal and neurochemical changes associated with pregnancy and parturition, and two groups of virgin females who did not. Within each of these parity groups, we assessed one group of animals who responded to pups and engaged in maternal behavior, and one group left without pups and therefore who do not respond maternally. We therefore consider the effects on gene expression of parity (and associated endocrine status), pup exposure, and the expression of maternal behavior, in addition to their interactions.

Here, we adopted a hypothesis-testing approach to microarray analyses, making predictions regarding expression of candidate genes that relate to specific hormones and neurochemicals in two prominent maternal brain structures, the MPOA and the medial amygdala (MeA), in animals that varied both in the degree of maternal behavior experience and in the extent to which they were hormonally primed. Within the MPOA and the MeA, we explored the pattern of expression of genes related to the hormones estrogen, progesterone, prolactin, oxytocin, and corticosterone; the neurotransmitters DA and 5HT; and the modulatory neuropeptides oxytocin, glutamate, opioids, GABA, and NOS. The genes of interest were, in general, genes associated with these maternally relevant signaling agents or their receptors.

Specific Predictions

Based on the extant literature, we made a number of predictions for the MPOA and the MeA. Although we ventured to make directional predictions, we did not know whether receptors reside on excitatory or inhibitory cells, therefore our weaker statistical hypotheses were all two-tailed. With respect to DA and OT and

their receptors/ligands/precursors, we expected an up regulation in the MPOA in pup-exposed, as opposed to non-pup-exposed, groups (PE vs. NPE), and a down regulation in the MeA. Furthermore, we predicted parity effects (postpartum vs. virgins) with estrogen, OT, and prolactin (and their receptors/ligands/precursors) in the MPOA. We expected opposite results in the MeA. Although we expected changes with corticosterone, GABA, glutamate, opioids, and NOS, this aspect was more exploratory, and we did not make predictions on directionality.

Method

Subjects

Subjects were 50- to 60-day-old female Sprague–Dawley rats, obtained from Charles River Farms (St. Constant, QC, Canada). Rats were individually housed in Plexiglas cages (26 × 43 × 21 cm) and provided with wood shavings as bedding. Room temperature and humidity were maintained at approximately 22 °C and 40–50%, respectively. Food (Purina Rat Chow) and water were made available ad libitum except during behavioral testing. Rats were maintained on a 12:12-hr light:dark cycle, with lights on at 8:00 a.m. Donor rats were also utilized and maintained under similar conditions and provided foster pups for maternal sensitization. In addition, three nonexperimental virgin female rats, controlled for age and strain, were also purchased from Charles River Farms and maintained under similar conditions for the purpose of providing reference RNA. Animal care and all experimental procedures used were in accordance with the guidelines provided by the Canadian Council on Animal Care and were approved by the Local Animal Care Committee at the University of Toronto.

Groups

Sixteen female virgin rats from four litters were assigned to one of four experimental conditions, such that each group contained one of four sisters: (a) postpartum females with pup experience (PP-PE), (b) postpartum females with no pup experience (PP-NPE), (c) maternally sensitized pup-exposed virgins (V-PE), and (d) control virgins (V-NPE).

Procedure

Maternal behavior. Animals in the postpartum condition were time-mated: vaginal smears were taken over several days at approximately 12:00 p.m. Once proestrus was established in the animals, they were placed with a sexually experienced male for 24 hr. Presence of spermatozoa in the vaginal smear was considered gestational day (GD) 0. Females were monitored around the time of parturition at 30-min intervals over 24 hr until the detection of birth. At this time, in the PP-NPE condition, newborn pups were removed immediately; dams were placed in clean cages and housed in a pup-deprived room for 24–36 hr. In the PP-PE condition, females were permitted to give birth undisturbed; following the birth of the entire litter, the litters of PP-PE dams were culled to three males and three females and placed with the dam in a clean cage for 24–36 hr.

Females in the V-PE condition were placed in clean standard cages and given shredded paper towel for nesting material 24 hr prior to the first sensitization. Virgin rats were exposed daily to four 2- to 8-day-old foster pups. The foster pups were replaced with newly fed pups at approximately 12:00 noon every day to ensure pup survival. Spot-checks were made 10 min and 2 hr following pup exchange to monitor maternal behavior. A V-PE rat was deemed maternal if all four pups were retrieved to the nest site for two consecutive days within 10 min of foster-pup replacement. The first day was designated as maternally sensitized day; expression of maternal behavior on 2 consecutive days ensured at least 24–36 hr of maternal experience (comparable to PP-PE dams). On average, virgin rats exhibit maternal behavior (to criterion) with 5–7 days of continuous exposure to foster pups (Rees, Panesar, Steiner, & Fleming, 2006). Females in the PP-NPE and V-NPE conditions were housed in a pup-deprived room and left undisturbed until the time of sacrifice. As much as possible, sister PP-PE and PP-NPE dams were sacrificed roughly at the same time (24–36 hr after parturition), and V-PE and V-NPE rats were sacrificed together (24–36 hr after V-PE's first maternally sensitized day).

Ten-min, undisturbed maternal observation tests were conducted in both PE conditions (PP-PE and V-PE) immediately prior to decapitation to ensure expression of maternal behavior. Frequency and duration of the following behaviors were recorded: (a) retrieval (picking up a pup in the mouth and carrying it to the nest site); (b) licking (both body licking and licking aimed at the anogenital region); (c) pup sniffing; (d) nest building (building a nest around the pups with shredded paper towel); (e) hovering (being over pups and simultaneously engaging in other behaviors); (f) crouching (arched back posture over pups); (g) mouthing (picking up and dropping a pup within a quadrant); (h) air sniffing; and (i) self-grooming.

Microarray, labeling, hybridization, and data analysis. The brains were removed and immediately immersed in RNAlater tissue storage and RNA stabilization solution (Ambion, Austin, TX). Coronal sections of 1 mm were cut using a vibratome (Leica, VT1000 S, Wetzlar, Germany) and tissue samples from the brain sites were extracted, using a 1000 μl RNase-free pipette tip (Ambion, Austin, TX). Bilateral samples were taken from the MPOA and the MeA, with borders specified by Paxinos and Watson (1986). Samples were stored in RNAlater in 1 ml aliquots at −80 °C until further processing. For reference RNA, tissue sections of the brain, heart, liver, kidneys, adrenal glands, ovaries, and muscles from three animals were taken and stored as above. RNA was extracted from the tissue using the Pinpoint Slide RNA Isolation System as per the manufacturer's instruction. The isolated RNA (8–9 μl) was separated into 1 μl, to measure the amount of RNA (ng/μl), and 8 μl for reaction time (RT)-polymerase chain reaction (PCR) amplification (for DNA amplification) and stored in −80 °C until further processing. RNA amplification was done using the Ovation Aminoallyl RNA Amplification and Labeling System (Version 1.1; NuGen Technologies, Inc, Brockville, ON, Canada) according to the company's protocol.

Indirect labeling of total RNA for microarray hybridization was done according to the protocol from the Canadian Drosophila Microarray Centre (University of Toronto at Mississauga, Canada). Each experimental sample was from one subject and was

hybridized to one array, paired with an aliquot of reference sample RNA. Sixteen arrays in total were hybridized (4 subjects/treatment × 4 treatments). Foreground and background intensities of experimental and reference samples were read on a scale from 2 to 100,000 counts. Only targets with expression in at least one treatment above 99.5% of background levels were retained. The remaining targets' experiment/reference spot intensities were $\log_2$ transformed and normalized using quantile normalization (Bolstad et al., 2003). Targets were identified by Ensembl ID, and where known RGD gene symbol; in cases where multiple targets for the same Ensembl ID or gene symbol existed only the target with highest mean expression was retained for analysis. This left 21,750 targets after applying the above steps.

Based on very specific hypotheses and predictions, we considered 150 primary genes of interest out of the 21,750, as we had persuasive physiological evidence for the importance of their products in maternal behavior. A list of the genes that showed significant differential expression and their functions are included in Supplemental Materials.

Statistical Methods

Statistical analyses used the R 2.8.1 statistical language, stats package (R Core Team, 2008). For each gene, significance of main and interaction effects was determined using analysis of variance (ANOVA) using the R functions aov and anova. With 16 subjects and a 2 × 2 factorial design, degrees of freedom (*df*) for *F* tests are 1,12 unless noted otherwise. Moreover, given the multiple tests that were done on approximately 150 genes, a multiple testing correction (Storey & Tibshirani, 2003) was applied. Genes are reported as significant if *p* value was $< .05$ based on 2 (exposure) × 2 (parity) ANOVA tests; however, we also report the *q* value, which indicates the false discovery rate or FDR. In general, a *q* value $< .20$ indicates that, at most, one out of five genes reaching statistical significance may actually occur by chance. We provide all significant *p* values for all the hypothesized genes with *q* value $< .35$, however, because (a) *q* value is also based on the expression level of the genes, and some, notably the dopamine-related genes, always exhibit very low expression levels, and (b) specific hypotheses are being tested relating to specific hormones and neurotransmitters, we did not want to reject true positives. In tabulated results we give both the original *p* value and the *q* value, indicating the FDR for all comparisons.

Results

Microarray Analyses, MPOA, and Pup Exposure (PE vs. NPE)

The first set of analyses considered the involvement of gene expression activated or inhibited by exposure to pups. *Df* for *F* tests are 1,12 unless noted otherwise. As shown in Figures 1–6, within the MPOA there were significant main effects of pup exposure for the dopamine-related genes, DRD4 ($F = 7.57$, $p < .015$, $q = .34$); and dopamine transporter, DAT (SLC6A3, $F = 6.32$, $p < .01$; $q = .26$; see Figure 1), the glucocorticoid-related gene (CYPX1B1a, CYP11B3, $F = 16.3$, $p < .002$, $q = .15$; see Figure 2), the opioid receptor gene (Opioid-R, Oprm1, $F = 15.5$, $p < .005$, $q = .13$; see Figure 3), and the GABA receptor gene (GABAbRid, GABRB1, $F = 5.22$, $p < .04$, $q = .09$; see Figure 4). All main effects in these cases revealed greater gene expression with pup exposure than without. Within the glutamate receptor gene (Glur1, Grm1, $F = 4.58$, $p < .05$, $q = .10$; see Figure 5), the GABA receptor gene (GABAT6, sLC12A2, $F = 8.13$, $p < .007$; $q = .14$; see Figure 4), the serotonin genes (5HT-hydroxylase, Tph2, $F = 10.1$, $p < .008$, $q = .19$; see

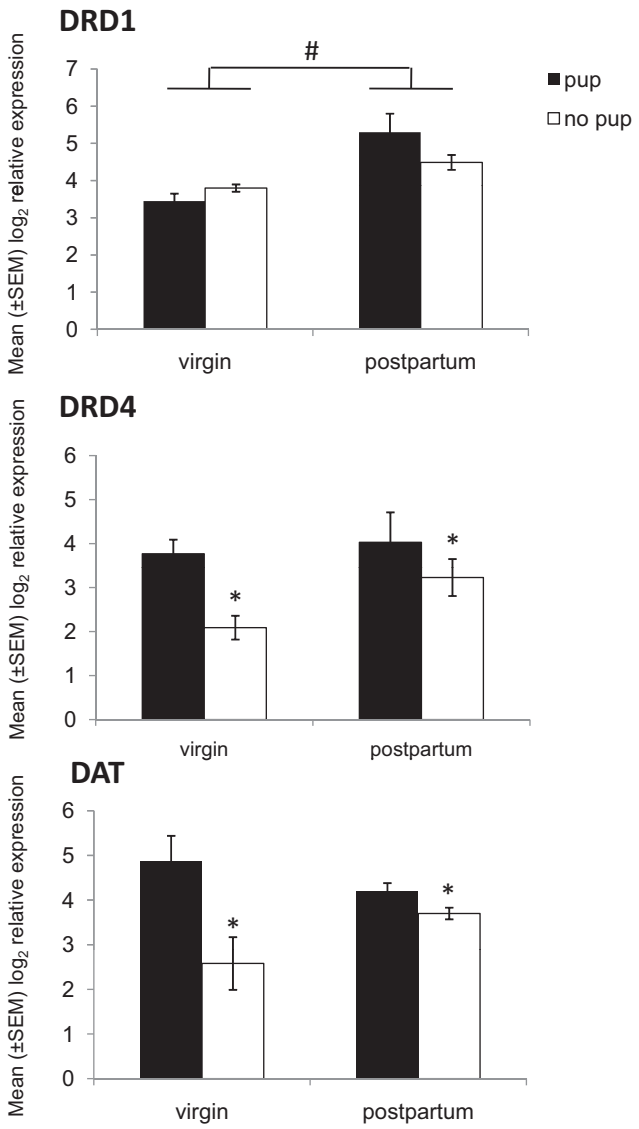

Figure 1. Mean (± *SEM*) $\log_2$ relative expression in DRD1, DRD4, and DAT. There were significant main effects for pup experience with DRD4 and DAT. Females with pup experience demonstrated higher levels of expression in these DA genes within the MPOA. There was a significant parity effect in the DRD1 gene; postpartum rats show higher levels of expression in this gene but this effect failed to fulfill the multiple testing criterion. Significance at the $p < .05$ level. Pound sign (#) represents main effect of parity; postpartum females have higher expressions of DRD1 than virgins. Asterisk (*) represents main effect of experience; females with pup experience have higher levels of gene expression.

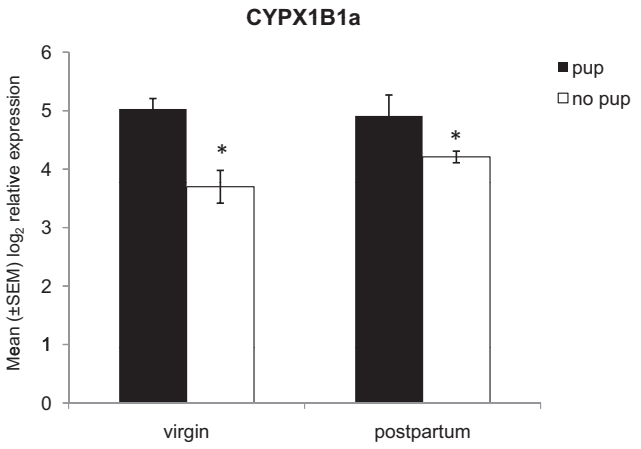

Figure 2. Mean (± *SEM*) $\log_2$ relative expression in the glucocorticoid-related gene CYPX1B1a. There was a significant main effect of pup experience. Females with pup experience demonstrated higher levels of expression in the CYPX1B1a gene in the MPOA. Significance at the $p < .05$ level. Asterisk (∗) represents main effect of experience.

Figure 6), and the GABAT6 genes, there was reduced expression in pup-exposed compared with the nonexposed animals.

Female Parity (PP vs. V)

Of the hypothesized genes, the genes that showed a main effect of parity were the opioid receptor genes. (*Df* for *F* tests are 1,12 unless noted otherwise; opioid-R, Oprm1, $F = 16.5$, $p < .001$, $q = .113$; see Figure 3), and the serotonin gene that regulates biosynthesis of serotonin (5HTR2A, $F = 14.3$, $p < .003$, $q = .29$; see Figure 6) where postpartum animals exhibited higher levels than virgins. The dopamine receptor gene, DRD1 gene showed a main

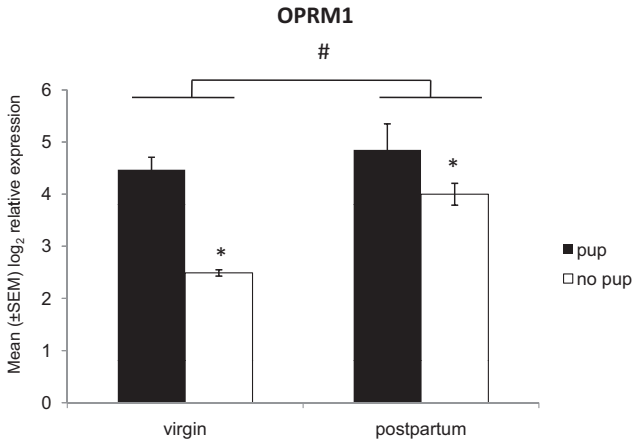

Figure 3. Mean (± *SEM*) $\log_2$ relative expression in the opioid receptor gene OPRM1. There was a significant main effect of pup experience. Females with pup experience demonstrated higher levels of expression in this gene in the MPOA. Significance at the $p < .05$ level. Pound sign (#) represents main effect of parity; postpartum females have higher expressions of gene expression than virgins. Asterisk (∗) represents main effect of experience; females with pup experience have higher levels of gene expression.

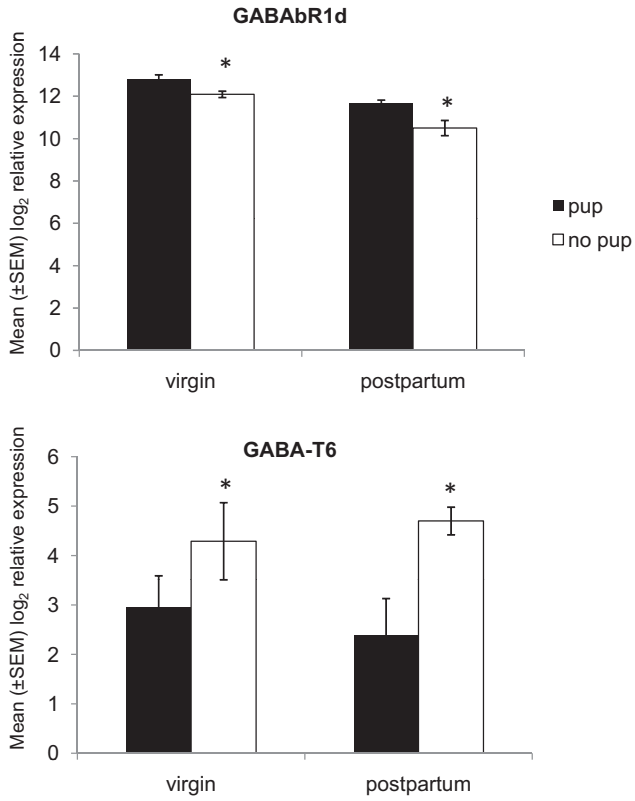

Figure 4. Mean (± *SEM*) $\log_2$ relative expression in the GABA receptor genes: GABAbR1d; and GABA-T6. There was a significant main effect of pup experience. Females with pup experience demonstrated higher levels of expression in the GABAbR1d gene, but lower expressions in the GABA-T6 gene in the MPOA. Significance at the $p < .05$ level. Asterisk (∗) represents main effect of experience.

effect of parity, however, it did not fulfill the multiple testing criterion (DRD1A, $F = 4.7$, $p < .04$, but $q = .53$; see Figure 1). We mention this only because we are careful about rejecting true positives, as we are testing specific hypotheses for DRD1, a gene which exhibits low expression levels.

Interactions Between Pup Exposure and Female Parity

Although there were a number of DA, estrogen, opioid, oxytocin, glucocorticoid, and GABA genes that achieved a significant Parity × Exposure interaction, attaining a $p < .05$–$.006$, with the exception of Glur1 and CORT-REG protein, the FDR was $> .35$ and hence, did not meet our acceptance criteria given multiple testing. Moreover, we had no a priori interaction hypotheses related to these genes. It should be noted, albeit it with caution, that for the opioid receptor gene, the GABA receptor gene and the oxytocin receptor gene, the direction of the interaction was that the postpartum animal with pups had the highest expression levels of all the groups. In contrast, for GLUR1 and CORT-REG protein, the interaction showed that the virgin groups had the highest levels of expression of all groups (GLUR1, the non pup-exposed virgins, $F = 5.64$, $p < .035$, $q = .30$; for the CORT-REG gene the pup exposed had the highest expression levels (star, $F = 4.6$, $p < .043$, $q = .21$).

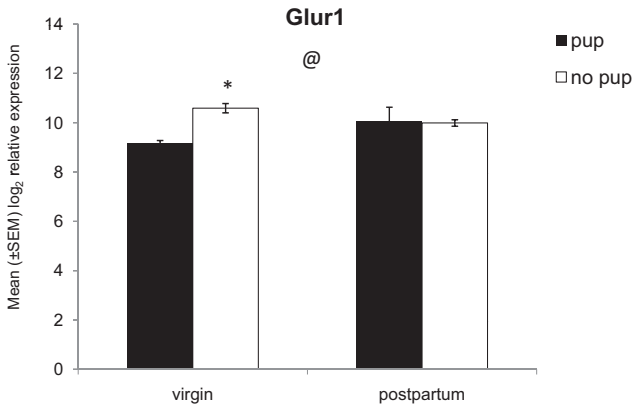

Figure 5. Mean (± *SEM*) $\log_2$ relative expression in the glutamate receptor gene (Glur1). There was a significant main effect of pup experience and interaction. Females with pup experience demonstrated lower levels of expression in this gene in the MPOA. Significance at the $p < .05$ level. "At" sign (@) represents a significant interaction.

Within the MPOA there were no significant changes as a function of exposure or parity or their interaction in the prolactin-receptor or oxytocin-receptor genes.

MeA

Pup-exposure. Only two MeA genes exhibited an effect of pup experience and they were within the glucocorticoid family. Only one met multiple testing criterion, (CORT-HSD2, Hsd11b2, $F = 14.5$, $p < .003$, $q = .26$); the other did not. For CORT-HSD2 mothers with pups had the highest levels of all groups.

Female parity and interactions of pup exposure and female parity. Although there were a few glucocorticoid genes that generated p values$<.05$ on the parity dimension, none of the genes met acceptable multiple testing criterion.

There were, however, a number of significant interactions between pup exposure and parity among estrogen synthesis genes (EST-HSD4, Hsd17b4, $F = 6.5$. $p < .023$, $q = .15$) and EST-Protein 1 (RGD1307526, $F = 4.8$, $p < .048$, $p = .21$), with virgins exposed to pups showing the highest expression levels of all the groups. There was also an interaction for three glucocorticoid synthesis genes, one of which reached the multiple testing criterion (CORT-HSD2, Hsd11b, $F = 6.32$, $p < .027$, $q = .24$), where postpartum animals with pups showed the highest expression of all groups.

To compare gene expression between brain sites, a 3-way ANOVA (site, exposure, parity) was undertaken on genes that were detectable at both sites. For all site comparisons $F(1, 12) = 7.0–20.0$, $p < .037–001$; $q < .03$). There were many genes that were expressed more in one site than in another, but no interactions of exposure or of parity with site. Genes that were expressed more in the MPOA than in the MeA included ESR1, $p < .001$, $q = 00$, and CYP 7B1, $p < .007$, $q = .00$. Genes that showed higher expression in the MeA were primarily the CORT genes CORT-REG (star), $p < .018$, $q = .02$, GR, Nr3c1, $p < .026$, $q = .037$), the GABA genes (GABAbR1a, Gabbr1, $p < .013$, $q = .016$; GABAbRid, Gabrb1, $p < .001$; $q = .00$) the glutamate gene (Grm1, $p < .004$, $q = .01$) and the serotonin gene (5HT-monoxygenase, Th, $p < .001$, $q = .00$). Of the 150 genes that we focused on in the present report, classes of genes with differential expression in the MPOA but undetectable in the MeA included dopamine genes, oxytocin genes, prolactin genes, or opioid genes. Of the 150 genes, there were none with detectable differences in expression in the MeA, but undetectable in the MPOA.

Discussion

We found that within the MPOA, the genes most clearly predicted to have expression patterns related to actual maternal behavior were associated with the DA and estrogenic systems. There were also groups of serotonin, and opioid-related genes that were expressed in new mothers and/or in animals when they are interacting with pups. In contrast, other genes, especially within the GABA systems were preferentially expressed in virgin animals. Within the MeA, the most notable pattern of gene expression was

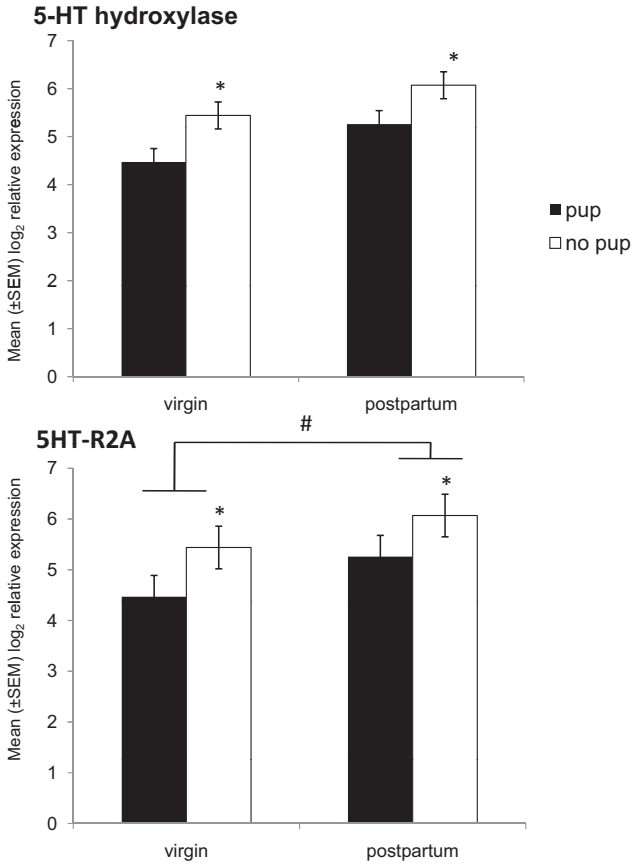

Figure 6. Mean (± *SEM*) $\log_2$ relative expression in the 5HT hydroxylase gene (Tph2) and the 5HT biothsynthesis gene 5HT-R2A. There was a significant main effect of pup experience in both genes. Females with pup experience demonstrated lower levels of expression in the 5HT hydroxylase gene and higher expressions in the 5HT-R2A gene in the MPOA. Significance at the $p < .05$ level. Pound sign (#) represents main effect of parity; postpartum females have higher expressions of 5HT-R2A than virgins. Asterisk (∗) represents main effect of experience; females with pup experience have lower levels of gene expression.

in the glucocorticoid gene family, where enhanced expression was found among postpartum animals that interacted with pups.

The category of genes that showed the largest *increases* in their expression in the MPOA in response to pup exposure was in the DA system and include the DRD4 and DAT genes. Although most of the dopamine receptor work relating to mothering has focused on the DRD1, DRD2, and recently DRD3 receptors systems, we found no effect of pup exposure in relation to any of these dopamine receptor subtypes. However, the DRD4 gene did differentiate experience groups. To our knowledge, the DRD4 receptor subtype has not been investigated in relation to maternal behavior. However this receptor gene shows increases in expression in animals with pup experience. This receptor has been implicated in integrating consumatory and appetitive (motivational) aspects of sexual activity (Succu et al., 2007). A similar system may be at play in maternal behavior, since these two reproductive behaviors are mediated by similar neurochemistry and neural circuitry. Further investigation is required to elucidate the role of the D4 receptor subtype in maternal responsiveness and care.

The DAT results in the present study are also in line with previous physiological findings and show a similar pattern to that of the DRD4 genes. The DAT, localized in the plasma membrane of axon terminals, serves to remove excess DA from the synaptic cleft, thereby ending the signal of this neurotransmitter (Mitchell & Gratton, 1992). Increased levels of DAT have in turn been associated with reduced extracellular DA (Baumann et al., 2002). Females that lick their pups less, show higher levels of DAT in the nucleus accumbens (NAC) compared with those that lick their pups more (Champagne et al., 2004). Finally, blocking DAT results in an increase in DA within the NAC and subsequently results in increased maternal licking by low licking mothers (Champagne et al., 2004). Interactions with pups in a maternal animal increases DA (Hansen, Bergvall, & Nyiredi, 1993; Olazábal, Abercrombie, Rosenblatt, & Morell, 2004), therefore, it is not surprising that with this pup-related increase in extracellular DA, the DAT gene also shows increases in expression (Mitchell & Gratton, 1992).

The present study also found that parturition resulted in the down-regulation in the 5HT1B and 5HT1F receptor genes in the MPOA. The 5HT5A receptor gene was up-regulated with pup-experience, whereas this experience increased the expression of the 5HT5B receptor gene in the MPOA but only in virgin females. Only more recently has the 5HT system been linked to maternal behavior. In primates, 5HT is implicated in anxiety arousal of mothers (Maestripieri, 2011) and lower levels of 5HIAA (5HT metabolite) are associated with more restrictive and rejecting maternal behaviors (Maestripieri et al., 2006). In rats, Hansen and colleagues (1993) reported an increase in the concentration of 5HIAA in the ventral striatum of females that were reunited with their pups after a period of separation. Furthermore, administration of 5HT agonists alters the peripheral release of oxytocin (Bagdy, 1995; Bagdy & Kalogeras, 1993), which has been implicated in both the onset of maternal behavior and lactation (Rosenblatt et al., 1988). Moreover, the central ventricular infusion of a 5HT receptor antagonist reduces maternal aggression without affecting maternal care (De Almeida & Lucion, 1994). The present study did not detect effects within these receptor subtype genes. Whether the down-regulation of this family of genes has a direct effect on maternal behavior or an indirect effect through the oxytocinergic system remains to be seen. However, we were unable to show differences as a function of pup-experience for the oxytocin or NOS genes.

The present study found effects contrary to our predictions in relation to pup-experience for expression of opioid and GABA related genes. For pup-exposure effects, all GABA and opioid-related genes showed greater expression in animals that were maternal and with pups, regardless of parity condition. This was opposite to the prediction for the neuropeptides, the opioid and GABA genes, whose gene products are thought to inhibit the expression of maternal behavior (GABA: Arrati, Carmona, Dominguez, Beyer, & Rosenblatt, 2006; Byrnes, Lee, & Bridges, 2007; opioids: Sobor et al., 2010; Miranda-Paiva, Nasello, Yin, & Felicio, 2001; Mann, Kinsley, & Bridges, 1991). In contrast, the only gene showing a decreased expression with pup exposure was the glutamate receptor gene. This gene showed the highest expression in virgin animals without exposure to pups.

In the MeA, we predicted that the direction of effects for OT, GABA, and glutamate would be opposite to their effects in the MPOA. These predictions were not confirmed, however, we did find higher levels of gene expression in the MeA for both GABA and glutamate genes. In terms of parity and experience interactions we found, contrary to our predictions, that the CORT-related genes were highest in postpartum pup exposed animals. These genes although classified as corticosterone genes, are within the family of annexin genes. Annexins have been implicated in many diverse functions such as cell division, apoptosis, growth regulation, and calcium signaling (Hayes & Moss, 2009). Furthermore, they mediate glucocorticoid action in the anterior pituitary and suppress the release of the adrenocorticotropic and corticotropin-releasing hormones (Taylor, Cowell, Flower, & Buckingham, 1993). Thus, it seems to be involved in the inhibition of the stress response. Moreover, the presence of annexin in the near-term placenta suggests it may be involved in the regulation and mediation of parturition (Challis et al., 2002). However, to our knowledge, annexin has not yet been studied in relation to maternal care, and thus these results lay down a foundation for the further investigation of the role of this versatile protein in maternal behavior.

Where they occurred, the counterintuitive effects that we found, point out a clear limitation in our interpretation of enhanced or reduced gene expression. Without knowing whether the neurons on which the different receptors reside are excitatory or inhibitory, the functional "meaning" or effect of the existence of high versus low levels of receptors cannot be discerned. High levels of opioid receptors and hence increased sensitivity to opioid ligands, on an excitatory neuron could have a depressing effect on activity of that neuron, whereas expression of the same receptors on an inhibitory neuron, may have a disinhibitory effect and hence be excitatory.

Despite clear experimental evidence that oxytocin, prolactin, and the gonadal hormones act on the MPOA to affect maternal behavior in the rat (see Numan et al., 2006; Shahrokh et al., 2010; for review, see Numan & Stolzenberg, 2009), we were unable to detect effects through microarray analyses in these genes. After correction for multiple testing, none of the associated hormone-related genes showed either parity or pup-exposure effects. Given the physiological evidence, the absence of an effect in relation to these receptor genes was surprising; it may be that the expression levels were so low that the sensitivity of the assay precluded their detection or that the changes were at the level of protein and not

RNA expression (Byrnes, Rigeo, & Bridges, 2002; Champagne, 2004; Parada et al., 2008; Li et al., 2004, 2005). Then again, lack of differential genetic expression may be because the genes for the primary gonadal and pituitary hormone receptors may be well expressed in all animals independent of the experience of gestation, parturition or maternal behavior experience. In fact, it may well be that since all animals have undergone many estrous cycles and earlier hormonal experiences, the receptors systems are poised to function in all female rats. What may then undergo more rapid change are the relevant neurotransmitters and neuropeptides that these hormones act upon or interact with.

One limitation of this study is that we did not monitor the estrous cycles of the female rats at the time of sacrifice. Although we sacrificed sister PP-PE and PP-NPE dams roughly at the same time (24–36 hr after parturition), and V-PE and V-NPE rats were sacrificed together (24–36 hr after V-PE's first maternally sensitized day), it would have been worthwhile to covary the estrous phase with our genetic profiles. Another limitation of our experimental design is that the V-PE rats (sensitized to become maternal over multiple days) had longer exposure to pups than the PP-PE rats that only experienced pups postpartum. It is possible that changes in the sensory environment during pup sensitization contributed to some of the changes seen in gene expression; however, we are unable to comment on the extent of this contribution. Given our sample size, it is not possible to correlate genetic expression with the latency to become maternal in sensitized rats. In future, it would be interesting to manipulate sensory environment by comparing rats at different stages of sensitization (initial exposure vs. partially or fully maternal, e.g.) to further separate the effects of mere pup exposure and expression of complex maternal behavior.

Although the genes examined in the present study need to be understood in greater detail in terms of their biochemical actions, the arrays can identify a candidate gene list of transcripts involved in maternal behavior and the factors that surround it. Activation of these genes, whether through up or down regulation, reveals the diverse effects of parturition and maternal responsiveness on cellular transcript levels.

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