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Experience-facilitated improvements in pup retrieval; evidence for an epigenetic effect

Danielle S. Stolzenberg, Ph.D., Jacqueline S. Stevens, and Emilie F. Rissman, Ph.D.

University of Virginia School of Medicine, Department of Biochemistry and Molecular Genetics, 1340 Jefferson Park Avenue, Charlottesville, VA 22908

Abstract

The quality and quantity of maternal care received during infancy is highly predictive of successful infant development. It has been well established, primarily in rats, that the combination of hormonal and infant stimuli at birth modifies neural circuits that regulate maternal responsiveness. During subsequent interactions, infant stimuli are more likely to elicit rapid maternal responsiveness. Some species, such as humans, can display maternal care in the absence of the endocrine events of pregnancy and birth. Similarly, virgin C57BL/6J female mice, display maternal care toward infants, and experience with infants elicits long-lasting increases in maternal care. We hypothesized that these experience-induced changes in behavior may be mediated by chromatin modifications, which in turn change expression of genes that promote maternal care. One site of action is the medial preoptic area (MPOA). To test our hypothesis we treated virgin female mice with sodium butyrate, a histone deacetylase inhibitor. This treatment potentiated maternal responsiveness as well as the expression of several genes: estrogen receptor β (*Esr2*), oxytocin (*Oxt*), and cyclic AMP response element binding protein (CREB) binding protein (*Crebbp*, a histone acetyltransferase) in the MPOA. These data suggest that experience induces high levels of maternal care via epigenetic modifications.

Keywords

maternal experience; epigenetics; sodium butyrate; HDAC inhibition; oxytocin; CBP

Introduction

In humans and other mammals, experience with infants has substantial effects on the quality of subsequent maternal care, which in turn affects infant development. In human mothers, more contact with infants during the first hours-days postpartum is associated with increased maternal responding, reduced infant crying, and an increase in secure infant attachment (Bystrova et al., 2009; Erlandsson et al., 2007; Kennell and Klaus, 1998; Klaus et al., 1972). For at risk mothers, greater infant contact is significantly correlated with a decreased incidence of child abuse/neglect (Buranasin, 1991; O'Connor et al., 1980). Therefore, understanding how experience with infants can produce changes in maternal care is essential

Correspondence should be addressed to: Dr. Danielle S. Stolzenberg, danielle.stolzenberg@gmail.com, Telephone (434) 982-4742, Fax (434) 243-8433.

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Conflict of Interest

The authors declare no conflicts of interest.

for understanding how these mechanisms might fail in mothers that fail to bond with their infants.

The mechanisms through which mother-infant interactions act on the brain to alter subsequent maternal responsiveness have been best characterized in rats, in which the combination of hormonal and infant stimuli at birth permanently enhances maternal responsiveness (Fleming and Korsmit, 1996; Fleming et al., 1999; Numan, 2006). For example, postpartum rats are highly responsive to pups and will learn to press a lever or traverse a novel environment to retrieve pups back to the nest (Fleming, 1994; Lee et al., 2000; Stern and Mackinnon, 1976). Even in the absence of continued hormone or infant exposure, female rats show long-lasting changes in maternal responsiveness (Bridges, 1975; Bridges, 1977; Bridges, 1978; Orpen and Fleming, 1987; Orpen et al., 1987; Scanlan et al., 2006).

The medial preoptic area (MPOA), is the critical neural site that responds to both hormonal and sensory inputs from pups and regulates behavior (Arrati et al., 2006; Fleming et al., 1983; Gray and Brooks, 1984; Jacobson et al., 1980; Kalinichev et al., 2000; Lee et al., 2000; Lee and Brown, 2007; Numan, 1974; Numan and Callahan, 1980; Numan et al., 1988; Numan et al., 1977). The MPOA undergoes a variety of changes pre- versus post-partum that promote maternal responsiveness (Afonso et al., 2009; Febo et al., 2005; Fleming and Korsmit, 1996; Kim et al., 2010; Kuroda et al., 2007; Meddle et al., 2007; Numan and Numan, 1994, 1995, 1997; Numan et al., 1998; Seifritz et al., 2003; Stack et al., 2002). Thus, interaction with infants in the context of these changes, likely modifies the MPOA such that during subsequent interactions, infant stimuli come to elicit maternal responsiveness more effectively.

However, not all species rely on the hormonal stimulation of birth to respond to infants. For example, when foster pups are scattered in the home cage of virgin laboratory mice, they respond to pups (retrieve, lick, crouch) within 15 minutes (Calamandrei and Keverne, 1994; Gandelman, 1973a, b; Gandelman and Vom Saal, 1975; Kuroda et al., 2008; Larsen et al., 2008; Leussis et al., 2008; Lucas et al., 1998; Mann et al., 1983; Noiro, 1972; Okabe, 2011; Stolzenberg and Rissman, 2011; Thomas and Palmiter, 1997). We have recently shown that experience with pups can initiate and sustain maternal responsiveness in a novel T-maze (high levels of maternal responsiveness) in virgin C57BL6/J mice. Moreover, behaviors displayed by virgins are not significantly different from postpartum females (Stolzenberg and Rissman, 2011). In addition, only 4 days of experience (for just 2 hours/day) with pups are required for females to show high levels of maternal responsiveness, whereas mice with 50% less pup experience (2 hours/day for 2 days) do not show this experience-induced increase in high levels of maternal responsiveness.

A critical question then is how this subtle difference in maternal experience can induce high levels of maternal responsiveness. We hypothesize that, in the absence of pregnancy and parturition, multiple experiences with infants may allow the transcription of genes that are typically regulated by the combination of hormonal stimulation and mother-infant interaction at birth to be activated in virgin mice. We speculated that epigenetic control of gene expression might contribute to the acute regulation of gene expression in response to experiences (Sweatt, 2009). One mechanism through which experience-dependent behavioral modifications are consolidated is epigenetic histone acetylation. Addition of acetyl groups, by histone acetyltransferases (HATs), to the histone proteins around which DNA is wrapped increases the sensitivity of DNA to transcriptional regulation.

In order to explore these hypotheses, we used a histone deacetylase inhibitor (HDAC inhibitor), sodium butyrate (SB), which inhibits HDAC activity and increases histone

acetylation (Rooszendaal et al., 2010). In the first experiment we examined whether HDAC inhibition would potentiate the effects of maternal experience on subsequent maternal behavior. In experiments 2 and 3 we examined the specificity of SB effects on maternal responsiveness. Finally, as a first step toward addressing how HDAC inhibition might mediate effects, we asked whether SB upregulated the expression of genes that are known to be associated with maternal experience.

Methods and Materials

Subjects and drug treatment

All mice were C57BL/6J virgin nulliparous females (60–100 days of age), naive to pups (except for their own littermates). Sodium Butyrate (SB; Sigma-Aldrich, MO) was dissolved in sterile water. The drug was diluted to a dose of 8mg/ml in the drinking water. Control mice received the vehicle (water) in the drinking water. Oral administration of SB at this dose increases histone acetylation in the hypothalamus (including MPOA) (Bonthuis et al., 2011). SB treatment began 10 days prior to testing and continued throughout testing (Minamiyama et al., 2004). All mice were single-housed during this time. Daily drinking was monitored for all animals. A separate group of C57BL/6J mice, drinking normal water, served as foster dams that provided stimulus pups. Mice were housed on a 12h reverse light cycle and given food (diet # 7912; Harlan Tekland, Indianapolis, IN) and water ad libitum. Behavioral testing occurred during the dark phase of the light/dark cycle under dim red light. All procedures were in compliance with the University of Virginia Animal Care and Use Committee.

Behavioral Procedures

Home cage maternal behavior testing—Twenty-four hours prior to the start of behavioral testing, each mouse was given a quarter of one nestlet. Stimulus pups were obtained from a group of donor mothers bred in advance. Pups from multiple mothers were collected together in an empty cage. The cage was placed on a heating pad until all the pups were randomly distributed to experimental females (no more than 15 minutes). At the start of each 2 hour test, 4 stimulus pups (2–7 days old) were scattered in the cage (Stolzenberg and Rissman, 2011). Latencies to retrieve each pup to the nest, group all pups inside the nest, and crouch over all pups inside the nest were recorded during the first 15 minutes. Pup retrieval was defined as picking a pup up in the mouth and transporting it to the nest. Females that did not retrieve during the first 15 minutes were assigned a latency of 900 seconds for statistical purposes; however note that all females had retrieved pups to the nest within 45 minutes of pup presentation. Therefore, during the last 15 minutes of the first test hour, behaviors toward all pups in the nest were recorded (licking, crouching, off nest) were recorded every 15 seconds. During the second test hour, behaviors were recorded every 3 minutes. At the end of the 2-hour exposure, pups were removed and returned to lactating donor females (not necessarily their biological mother).

Novel T-maze maternal behavior testing—The walls and floors of the T-maze apparatus ($67.3 \times 11.4 \times 8.3$ cm) were clear Plexiglas upon which a removable wire mesh top was fitted. The vertical runway measured 48.3 cm in length and opened into a horizontal runway that measured 67.3 cm in length. An $11.4 \text{ cm} \times 12.7 \text{ cm}$ goal box was attached to the end of the vertical runway which could be closed off from the rest of the T-maze by a clear Plexiglas guillotine door. Three stimulus pups were scattered in the horizontal arm of the Plexiglas T-maze. At the start of the retrieval test, each female was placed into the goal box of the T-maze with her nest material. After a 10-minute habituation period, the Plexiglas door was removed and the 15-minute pup retrieval test began. Latencies to emerge from the goal box (all four paws), sniff a pup, and retrieve each pup to the goal box were recorded.

The test ended after 15 minutes, or when the female had retrieved all 3 pups to the goal box. Females that did not retrieve pups during the test were assigned a latency of 900 seconds for statistical purposes.

Novel T-maze inanimate object testing—Using the same test described above, three rubber toys, the same size and shape as a pup, were scattered in the horizontal arm of the maze.

Elevated plus maze—At the start of the test, the mouse was placed in the center of the elevated plus maze (EPM), facing an open arm, and allowed to explore the maze for 10 minutes. The following behaviors were recorded: time spent in the open arms (s), time spent in the closed arms (s), and number of times the mouse crossed from one arm to the other. An animal was considered to be in an arm if all four paws were inside the arm.

Steroid Hormone Assay

Blood was collected from female mice treated with SB or Water at the end of behavioral testing, and after centrifugation plasma was frozen. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (supported by the Eunice Kennedy Shriver NICHD/NIH (SCCPIR) Grant U54-HD28934) determined plasma concentrations of estradiol and progesterone (Calbiotech, Inc.) by ELISA and radioimmunoassay, respectively. The detectable range for estradiol and progesterone was 3.0–300.0 pg/ml and 0.1–12 ng/ml, respectively. All estradiol samples and nearly half of the progesterone samples were run in duplicate. The intra-assay variability for the estradiol and progesterone assay was $3.73 \pm 1.82\%$ and $0.14 \pm 0.12\%$, respectively.

Quantitative real-time PCR

Mice were briefly anesthetized with isoflurane and euthanized by cervical dislocation. Brains were immediately removed, frozen, and later sectioned (120 microns) on a cryostat, and mounted onto slides. The MPOA (Bregma 0.26 to –0.58, Franklin and Paxinos Mouse Brain Atlas) was dissected out using a tissue punch. Total RNA was isolated using an RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The quantity and quality of the RNA was determined using a NanoVue™ Spectrophotometer. The cDNA templates were prepared using an AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The ABI StepOnePlus real-time PCR system was used to perform qPCR. The following TaqMan® Gene Expression assays from (Applied Biosystems, Carlsbad, CA) were used to detect PCR products of interest: CREB binding protein (*Crebbp*, Mm01342452_m1), estrogen receptor α (*Esr1*, Mm00433149_m1), estrogen receptor β (*Esr2*, Mm00599821_m1), oxytocin (*Oxt*, Mm00726655_S1), oxytocin receptor (*Oxtr*, Mm01182684_m1), arginine vasopressin (*Avp*, Mm00437761_g1), and arginine vasopressin receptor 1a (*Avpr1a*, Mm00444092_m1). Target and endogenous control genes were measured in triplicate for each cDNA sample during each real-time run to avoid inter-sample variance. All samples were normalized to $\beta 2$ microglobulin (Mm00437762_m1) and genes of interest were quantified relative to a sample from the 2 days of experience + Water group (Relative quantification = 1). Normalization and quantification of the genes of interest and $\beta 2$ microglobulin mRNA were analyzed with StepOne™ Software using the comparative cycle thresholds method (C_T) method.

Statistical Analysis

All data were analyzed using NCSS (2007, Kaysville, UT). Maternal responsiveness in the home cage during Test Days 1–2 was analyzed by a mixed two-way ANOVA (Treatment X

Test Day) with repeated measures on the second factor, followed by Newman-Keuls post hoc tests for planned comparisons between treatment groups on each Test Day. Cohen's d (effect size) was calculated for each difference between the two treatment groups on test days 1–2. Behavioral data that violated assumptions of normality and/or homogeneity of variance were analyzed by nonparametric statistics (Mann-Whitney U Test). Percent of females retrieving pups on the T-maze was analyzed by a Fisher's exact probability test. One-way ANOVAs were used to analyze relative quantification between treatment groups for each gene, followed by Newman-Keuls post hoc tests for planned comparisons and samples that were identified as outliers by the Grubb's test were removed from statistical analysis (Burns et al., 2005). For all data, significance level was set at $p < 0.05$, two-tailed.

Design for Experiment 1

Mice were randomly assigned to the following groups: SB ($n=7$) or Water ($n=7$). All females received 2 consecutive days (2 hours/day) of maternal experience, during which maternal behaviors in the home cage were recorded. On the following day, 24 hours after the last experience, mice were tested for pup retrieval on the novel T-maze.

Design for Experiment 2

Given that SB treatment had some effects on pup retrieval in the home cage on the first pup exposure (Table 1), we tested the hypothesis that HDAC inhibition induced maternal responsiveness on the T-maze by affecting pup retrieval directly, rather than enhancing some aspect of maternal *experience* on pup retrieval in the T-maze. A separate group of virgin mice received 10 days of SB ($n = 8$) or Water ($n = 7$) treatment and were then tested for maternal responsiveness on the T-maze immediately, without previous experience with pups.

Design for Experiment 3

To address the behavioral specificity of sodium butyrate effects on maternal care we used a separate group of mice. These mice were also treated with SB ($n= 10$) or Water ($n= 10$) for 10 days prior to and throughout testing, and given 2 consecutive days of maternal experience before EPM and novel T-maze testing.

Design for Experiment 4

We have previously reported that long-lasting changes in maternal responsiveness of virgin mice require 4 days (2 hours/day) of maternal experience. The results of Experiment 1 showed that treatment with SB reduced the amount of maternal experience required to promote pup retrieval on the novel T-maze. To address whether SB treatment also reduced the amount of maternal experience required to affect gene expression in the MPOA, we compared gene expression between virgin mice with 4 days of experience, 2 days of experience, and 2 days of experience with SB.

Results

Experiment 1: Effects of HDAC inhibition on maternal experience-dependent maternal responsiveness

Upon initial exposure to pups in the home cage, SB treated females were significantly faster to retrieve all pups to the nest [main effect of Treatment $F(1,12)= 6.81$, $P = 0.02$] when compared with Water treated control females (Table 1). Post hoc analyses revealed that SB treated females were significantly different than Water treated females on Test Day 1. Although there were no significant effects of SB treatment on latency to group all pups inside the nest [$F(1,12)= 2.67$, $P = 0.13$], or crouch over pups in the nest [$F(1,12)= 2.59$, $P =$

0.13], note that the effect size is rather large for these differences on Test Day 1 ($d = 0.98$, $d = -1.07$, respectively; Table 1). Similarly, on Test Day 2, although latency to retrieve pups to the nest is not significantly different between treatment groups, note that this difference has a large effect size ($d = 1.05$). All females retrieved all pups to the nest faster [main effect of Test Day, $F(1,12) = 7.54$, $P = 0.02$], grouped pups inside the nest faster [main effect of Test Day, $F(1,12) = 5.48$, $P = 0.04$], and crouched over pups more quickly [main effect of Test Day, $F(1,12) = 14.03$, $P = 0.003$] on the second test day. There were no significant effects of Treatment or Test Day on frequency of licking [$F(1,12) = 0.02$, $P = 0.89$; $F(1,12) = 0.01$, $P = 0.9$, respectively], crouching [$F(1,12) = 2.6$, $P = 0.13$; $F(1,12) = 0.24$, $P = 0.63$, respectively], or total contact with pups [$F(1,12) = 0.44$, $P = 0.52$; $F(1,12) = 0.04$, $P = 0.85$, respectively] in the home cage during the 15 minute observation. Analysis of the second hour of pup exposure did not reveal any significant differences in the frequency of licking, crouching, or total contact with pups (not shown) between SB and Water treated females.

On the following day, in the novel T-maze, the percentage of mice retrieving all 3 pups on the T-maze was significantly higher in the SB group compared with the Water group, 85% versus 14%, respectively (Fisher's exact probability test, $P = 0.029$, Fig. 1A). The median latency for SB treated females to retrieve the first pup was 310 seconds, which was significantly faster than the 900 second median latency of control females (Mann-Whitney U test, $U = 42$, $P = 0.02$; Fig. 1B). Similarly the median latencies for SB treated females to retrieve the second (386 versus 900 seconds) and third pups (429 versus 900 seconds) were significantly shorter than the Water group ($U = 43.5$, $P = 0.01$, $U = 44$, $P = 0.008$, respectively). SB treated females retrieved significantly more pups (median = 3) than Water treated females (median = 0, $U = 41$, $P = 0.02$; Fig. 1C). There were no significant differences in median latency to approach and sniff the first pup on the T-maze between the two groups (Mann-Whitney U test, $U = 36.5$, $P = 0.12$; Fig. 1D).

Experiment 2: Effects of HDAC inhibition on maternal responsiveness in pup-naive mice

Pup-naive mice were not responsive to pups on the novel T-maze, with or without SB treatment (Table 2). There were no significant differences between groups in median latencies to emerge from the goal box (Mann-Whitney U Test, $U = 37.5$, $P = 0.27$), sniff the first pup ($U = 35$, $P = 0.42$), retrieve the first pup ($U = 31.5$, $P = 0.35$), or median number of pups retrieved ($U = 31.5$, $P = 0.35$).

Experiment 3: Effects of HDAC inhibition on exploratory behavior and circulating steroid hormones

No significant differences were detected between SB and control females on any measures in the EPM or the novel T-maze when an inanimate object was present (Table 3). Time spent on the open arms was not significantly different between SB treated and Water females [$t(18) = 0.07$, $P = 0.94$]. Similarly, there were no differences in time spent in the closed arms [$t(18) = 1.23$, $P = 0.23$]. There were also no differences between groups in the number of times a female crossed between arms [$t(18) = -0.07$, $P = 0.95$]. SB treated females did not emerge from the goal box of the T-maze or sniff an inanimate object on the T-maze faster than Water females [$t(18) = -0.07$, $P = 0.93$; $t(18) = 0.9$, $P = 0.37$, respectively]. Plasma concentrations of estradiol [$t(8) = -0.46$, $P = 0.65$] and progesterone [$t(18) = -0.61$, $P = 0.54$] were not significantly different between SB and Water females (Table 4).

Experiment 4: Effects of HDAC inhibition on gene expression

Analysis of gene expression in MPOA revealed a significant main effect of treatment on the expression a several genes (Fig. 2): *Crebbp* [$F(2,19) = 6.04$, $P = 0.009$], *Esr2* [$F(2,19) = 4.67$, $P = 0.02$], *Oxt* [$F(2,17) = 12.55$, $P = 0.0005$], *Avpr1a* [$F(2,20) = 3.53$, $P = 0.048$], *Avp*

[F(2,20) = 206.71, $P = 0.00000$] (Fig. 2). There was a trend for an increase in *Oxtr* mRNA [*Oxtr* F(2,15) = 3.43, $P = 0.059$]. No significant changes in *Esr1* were detected [*Esr1* F(2,18) = 0.7, $P = 0.5$]. Post hoc analyses revealed that 4 days of experience + Water was associated with an increased expression of *Crebbp*, *Esr2*, *Oxt*, *Avpr1a*, and *Avp* relative to 2 days of experience + Water. *Oxt* and *Avp* expression were also significantly higher in mice with 4 days of experience + Water relative to females with 2 days of experience + SB. SB treatment significantly increased the expression *Crebbp*, *Esr2*, and *Oxt*, relative to animals with 2 days of experience + Water ($p < 0.05$). SB treated females with 2 days of experience were not significantly different from animals with 4 days of experience + Water in the expression of *Crebbp* and *Esr2* ($p < 0.05$).

Discussion

Here we report that treatment with SB, an HDAC inhibitor, amplified the effects of maternal experience, both on maternal responsiveness in a novel environment and on gene expression in the MPOA. We have previously reported that virgin female mice require 4 days (2hours/day) of maternal experience in order to show high levels of maternal responsiveness on a novel T-maze (Stolzenberg and Rissman, 2011). Thus, treatment with SB effectively reduced by 50% the amount of maternal experience required to promote maternal responsiveness to pups in this novel setting. Our results also indicate that these maternal-experience dependent changes in maternal care are associated with an upregulation of gene expression (*Crebbp*, *Esr2*, *Oxt*, *Avp*, and *Avpr1a*) in the MPOA, and SB also reduced the amount of maternal experience required to promote the expression of 3 of these 5 genes (*Crebbp*, *Esr2*, and *Oxt*). To our knowledge, we are the first to report that experience with infants alone is associated with an upregulation of some of same genes that are typically elevated at birth (Champagne et al., 2001; Champagne et al., 2003; Gammie et al., 2005; McLeod et al., 2007; Meddle et al., 2007).

The results of the present study support the hypothesis that HDAC inhibition facilitates maternal experience-dependent changes in maternal responsiveness. However, SB treatment also accelerated pup retrieval in the home cage on the first pup exposure (Table 1). Interestingly, although SB treatment facilitated pup retrieval in the home cage in pup-naïve mice, it was not capable of inducing pup retrieval on the novel T-maze in pup-naïve mice. One possibility is that for virgin mice the environment plays a role in the valence of pup stimuli. For example, in a familiar environment, sensory cues from pup stimuli might be neutral. In this case, SB treatment might potentiate the ability of these cues to elicit retrieval behavior. Whereas in a novel environment, sensory cues from pup stimuli might be aversive to pup-naïve mice. Therefore, in order to retrieve pups on the T-maze, virgin mice must overcome an avoidance of pup stimuli, similar to the process of sensitization in rats. In this case, SB treatment alone would not affect pup retrieval.

It is well known that treatment with various HDAC inhibitors can modulate learning and memory (Alarcon et al., 2004; Korzus et al., 2004; Levenson et al., 2004; Malvaez et al., 2010; Roozendaal et al., 2010; Stefanko et al., 2009; Vecsey et al., 2007; Yeh et al., 2004), sexual behavior (Bonthuis et al., 2011), motivation (Laplant and Nestler, 2010), depressive-like behavior (Covington et al., 2009; Gundersen and Blendy, 2009; Zhu et al., 2009), and stress responses (Mifsud et al., 2011). A relevant question then is whether the facilitatory effect of SB on maternal responsiveness is specific to this behavior. First, to ask if SB facilitated retrieval on the novel T-maze in a non-specific manner we used inanimate objects, the size and shape of a pup, instead of pups, on the T-maze (Table 3). Females did not retrieve these objects. Second, SB might reduce anxiety and increase tolerance for pups, rather than directly enhance experience-dependent maternal care. We tested females in the elevated plus maze to evaluate anxiety and found no significant effect of SB treatment on

time spent in the open or closed arms of the maze (Table 3). Third, because ovarian hormones are involved in many aspects of maternal behavior we measured circulating levels of estradiol and progesterone (Table 4) at the time testing. SB treatment did not significantly alter plasma concentrations of estradiol or progesterone as compared with Water-treated controls. Finally, because of the nature of our testing protocol (random distribution of pups) it is highly unlikely that differences in retrieval between experimental groups could be due to recognition of specific pups. Taken together, the results of the present study indicate that HDAC inhibition did not affect exploratory behavior in a novel environment or circulating hormone levels.

The present data show that 4 days (2hours/day) of pup experience (which induces high levels of maternal responsiveness) is associated with increased expression of *Esr2*, *Oxt*, *Avp*, and *Avpr1a*. These findings are compatible with the idea that experience with pups might substitute for pregnancy hormone stimulation by affecting some of the same genes that are typically upregulated at birth (Champagne et al., 2003; Ehret and Buckenmaier, 1994; Giordano et al., 1989; Meddle et al., 2007). We speculate that in the absence of pregnancy hormones, pup experience alone might affect gene expression through the induction of intracellular signaling cascades, which can phosphorylate transcription factors and turn on gene expression. For example, activation of CREB and extracellular signal related kinase (ERK) is part of the transcriptional pathway that supports maternal responsiveness in both postpartum and virgin female mice (Jin et al., 2005; Kuroda et al., 2007). Further, subsequent activation of CBP by these cell surface signals could result in transcriptional regulation and histone acetylation in the promoter regions of genes with estrogen responsive elements as well as those with cyclicAMP responsive elements (Bouchal et al., 2011; Kim et al., 2001; Levenson and Sweatt, 2005; Riccio, 2010). The finding that treatment with an HDAC inhibitor potentiated the expression of some of these same genes (*Crebbp*, *Esr2*, and *Oxt*) is in agreement with our hypothesis that experience-dependent effects on gene expression may be regulated by epigenetic modifications. Our data are also consistent with reports that HDAC inhibitors affect estrogen receptor gene expression as well as estrogen receptor transcriptional activity *in vitro* (Cheung et al., 2003; Duong et al., 2006). In addition, while HDAC inhibition has been found to affect estrogen signaling in both the presence and absence of ligand (estradiol), behavioral effects of the HDAC inhibitor SB require intact ovaries (Bonthuis et al., 2011; Zhu et al., 2009).

Importantly, whereas SB requires ovaries for its actions on maternal responsiveness (unpublished observations), experience effects on high levels of maternal responsiveness do not (Stolzenberg and Rissman, 2011). We have demonstrated that 4 days (2hours/day) of maternal experience can induce maternal responsiveness on the T-maze in ovariectomized mice that are not capable of synthesizing estradiol. Therefore, a critical question that will be resolved by ongoing research is whether the same genes are associated with experience-dependent changes in maternal responsiveness in the complete absence of estradiol. In support of the possibility that the same genes are involved, note that the induction of estrogen receptor immunoreactivity in the MPOA is similar between pup-experienced (5 days) ovariectomized and intact outbred mice (Ehret and Buckenmaier, 1994).

At present, it is unclear whether the genes we found associated with maternal experience are necessary for experience-dependent effects on high levels of maternal responsiveness in mice. For example, whether mice lacking these genes (*Crebbp*, *Esr2*, *Oxt*, *Avp*, or *Avpr1a*) would show experience-dependent changes in maternal responsiveness has not been directly tested. There is however ample evidence to support the role that these genes play in maternal care. For example, mice lacking functional estrogen receptor α show deficits in pup retrieval during a 15-minute test in the home cage (Ogawa et al., 1998). Mice lacking estrogen receptor β reportedly show normal reproductive behavior, although maternal responsiveness

was not tested directly (Ogawa et al., 1999). Further, individual differences in licking and grooming of pups is linked with expression of estrogen receptors in MPOA. Although there is some evidence that an increase in *Esr2* expression in the MPOA is associated with increased maternal behavior during the postpartum period (Champagne et al., 2001; Champagne et al., 2003; Gammie et al., 2005; McLeod et al., 2007; Meddle et al., 2007), the role of *Esr1* in mediating the quality of maternal care has been described more extensively (Champagne et al., 2001; Champagne et al., 2003).

Estrogen receptors regulate many other genes including, *Oxt*, *Oxtr*, *Avp*, and *Avpr1a*, which also play an important role in maternal behavior. The increase in oxytocin action at oxytocin receptors in the MPOA at birth has been linked to mother-infant bonding in a variety of animals (Champagne et al., 2001; Kendrick et al., 1997; Numan, 2003; Pedersen et al., 1994). Further, oxytocin plays a critical role in the onset of maternal behavior (Fahrbach et al., 1986; Pedersen et al., 1994) and the consolidation of maternal experience in rats (D'Cunha et al., 2010). Mutations in *Oxt* or *Oxtr* gene have been found to negatively affect pup retrieval in nulliparous virgin mice (Macbeth et al., 2010; Pedersen et al., 2006; Takayanagi et al., 2005), particularly when females are tested in novel or stressful environment (Pedersen et al., 2006; Ragnauth et al., 2005). However, postpartum mice with these mutations show a normal onset of maternal behavior (Macbeth et al., 2010). Recent work has shown that vasopressin release and increased vasopressin 1a receptor binding in the MPOA is associated with maternal responsiveness of postpartum female rats (Bosch et al., 2010) and maternal memory in the medial amygdala (Nephew et al., 2009).

It has been beautifully documented that epigenetic regulation of ER expression is one mechanism through which early life experiences impact adult maternal behavior in rats (Champagne and Curley, 2008). The transmission of maternal responsiveness from mother to daughter has been found to be associated with the extent to which the regulatory region of the *Esr1* gene is methylated (Champagne et al., 2006), which in turn affects estrogen receptor α expression as well as the expression of the estrogen receptor α responsive gene, *Oxtr*. Here we report that the high level of maternal responsiveness induced by maternal experience is associated with greater expression of *Esr2*, but not *Esr1*, in MPOA. Further, maternal experience was associated with an upregulation of the estrogen receptor β responsive gene, *Oxt*, but not its receptor, which is regulated by estrogen receptor α (Patisaul et al., 2003). Although the present data do not reflect those patterns of gene expression that are influenced by early life experience and predisposed patterns of maternal care, it is possible that the mechanisms through which initial maternal experiences alter subsequent maternal responsiveness might be different. However, the fact that both of these patterns ultimately affect oxytocin action in the MPOA, suggests a potential parallel pathway through which dynamic epigenetic alterations allow mother-infant interaction to dynamically alter subsequent maternal care. In support of this idea, of the two estrogen receptor subtypes, HDAC inhibitors have more robust effects on estrogen receptor β expression and estrogen receptor β transcriptional activity in vitro (Cheung et al., 2003; Duong et al., 2006). Therefore, HDAC inhibition may allow estrogen receptor β to be equally as effective, or more effective than estrogen receptor α .

Although multiple factors can partially predict the quality of human maternal care (Benoit and Parker, 1994; Chapman, 2001; Pederson et al., 1998), there is ample evidence that even small increases in initial infant contact have the potential to change the course of mother-infant relationships (Buranasin, 1991; Bystrova et al., 2009; Kennell et al., 1974; Kennell and Klaus, 1998; Klaus et al., 1972; O'Connor et al., 1980). The present data are a first step toward uncovering the molecular mechanisms through which initial mother-infant interactions induce epigenetic alterations that sustain maternal care during this critical period. However, future work will resolve the extent to which histone acetylation is

associated with the increased expression of estrogen receptor β and CBP through the use of chromatin immunoprecipitation. Further, the extent to which these genes are necessary for maternal experience effects on maternal care is presently being resolved through the use of genetic mouse models.

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Research Highlights

- Maternal experience facilitates subsequent maternal care in C57/BL6J mice
- Experience-dependent effects are associated with increased gene expression in medial preoptic area
- A histone deacetylase inhibitor potentiated experience effects on behavior and gene expression

Figure 1.

The effects of the histone deacetylase inhibitor, sodium butyrate (SB), on maternal responsiveness in a novel T-maze after 2 days (2hours/day) of maternal experience 24 hours after last pup exposure (n's = 7/group). **(A)** Percentage of females in each group retrieving 1, 2, or 3 (all) pups on the T-maze. **(B)** Mean $\pm$ SEM and median (black bars) latencies in seconds (s) to retrieve each pup in the T-maze **(C)** Mean $\pm$ SEM and median (black bars) number of pups retrieved in the T-maze **(D)** Mean $\pm$ SEM and median latency to sniff a pup on the T-maze. *Significantly different from corresponding Water control group, Fisher's exact probability, ($p < 0.05$). **Significantly different from corresponding Water control group, Mann-Whitney U test, ($p < 0.05$).

Figure 2.

Sodium butyrate amplifies the effects of 2 days (2 hours/day) of experience on gene expression in MPOA. Females with 4 days of experience received 2 hours of experience/day. Mean $\pm$ SEM relative quantification of *Crebbp*, *Esr2*, *Oxt*, *Oxt*, *Avp*, and *Avpr1a* mRNA in MPOA 24 hours after last pup exposure (n's=5–8 mice/group). Groups with different letters are significantly different from each other for each gene ($p < 0.05$). Groups without letters were not significantly different. Abbreviations: *Avp*= vasopressin; *Avpr1a*= vasopressin 1a receptor; *Crebbp*= cyclic AMP response element binding protein binding protein; *Esr1*= estrogen receptor α ; *Esr2*= estrogen receptor β ; MPOA= medial preoptic area; *Oxt*= Oxytocin; *Oxt*= Oxytocin receptor; SB= sodium butyrate.

Table 1
Observed Maternal Behaviors in the Home Cage During 2 Daily Trials

Females were observed on two consecutive days for 2 hours on each trial. Latency data are expressed as Mean $\pm$ SEM Frequency of maternal behaviors are expressed as number of observations (out of 60 total) licking pups, crouching over pups, or in contact with pups during a 15 minute observation. Effect sizes (Cohen's d) were calculated by comparing the means and standard deviations between two treatment groups for each measure on each test day.

Group	N	Latency to Retrieve All Pups(s)	Latency to Group Pups Inside Nest (s)	Latency to Crouch (s)	Number of Licking events	Number of Crouching events	Number of Pup Contacts
Test Day 1							
SB	7	317 $\pm$ 84 [*]	343 $\pm$ 104	423 $\pm$ 91	19 $\pm$ 5	21 $\pm$ 8	50 $\pm$ 8
Water	7	617 $\pm$ 108	619 $\pm$ 108	687 $\pm$ 95	24 $\pm$ 5	21 $\pm$ 7	57 $\pm$ 2
Effect Size		d = 1.17	d = 0.98	d = -1.07	d = -0.37	d = 0	d = -0.45
Test Day 2							
SB	7	150 $\pm$ 32 [^]	236 $\pm$ 80	268 $\pm$ 77 [^]	24 $\pm$ 4	27 $\pm$ 5	60 $\pm$ 0.3
Water	7	337 $\pm$ 89 [^]	404 $\pm$ 93	274 $\pm$ 39 [^]	18 $\pm$ 3	8 $\pm$ 4	46 $\pm$ 5
Effect Size		d = 1.05	d = -0.73	d = -0.03	d = 0.64	d = 1.58	d = 1.49

^{*} Significantly different from Water treated group on Test Day 1, Newman-Keuls test, $p < 0.05$

[^] Significantly different from Test Day 1, Newman-Keuls test, $p < 0.05$

Table 2
The Effects of Sodium Butyrate on Maternal Responsiveness on the T-maze in Pup Naive Female mice

Maternal responsiveness on the T-maze in mice that are pup-naive. All data expressed as Median (Interquartile range). There were no significant differences between groups.

Group	N	Latency to Emerge from Start Box (s)	Latency to Sniff First Pup (s)	Latency to Retrieve First Pup (s)	Number of Pups Retrieved
Pup naïve + Water	7	25 (20–41)	55 (35–63)	900 (900–900)	0 (0–0)
Pup naïve + SB	8	30 (27.22–33.25)	58 (41.5–99.5)	900 (900–900)	0 (0–0)

Effects of Sodium Butyrate on Exploratory Behaviors on the Elevated Plus Maze and T-maze

Table 3

Activity on the elevated plus maze during the 10-minute test. All data are expressed as Mean $\pm$ SEM. There were no significant differences between groups on any measure.

Group	N	Open Arms (s)	Closed Arms (s)	Arm-Arm Crosses	Latency to Emerge from Goal Box	Latency to Sniff Inanimate Object
Water	10	94 $\pm$ 20	364 $\pm$ 12	34 $\pm$ 4	19 $\pm$ 3	51 $\pm$ 10
SB	10	93 $\pm$ 8	344 $\pm$ 11	35 $\pm$ 4	20 $\pm$ 2	41 $\pm$ 6

Table 4
Effects of Sodium Butyrate on Plasma Hormone Levels

Plasma levels of estradiol (n=5/group) and progesterone (n=10/group) are expressed as Mean $\pm$ SEM. There were no significant differences between groups.

Group	Plasma Estradiol (pg/ml)	Plasma Progesterone (ng/ml)
Water	15.04 $\pm$ 1.89	2.9108 $\pm$ 0.5
SB	16.58 $\pm$ 2.73	3.9386 $\pm$ 1.6