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Immediate and Enduring Effects of Neonatal Isolation on Maternal Behavior in Rats

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Abstract

Previously, we showed that neonatal isolation (1-hr isolation/day from dam, litter, and nest on PND 2-9) facilitates cocaine self-administration and increases extracellular dopamine responses in ventral striatum after stimulant administration in adulthood. Recent studies suggest that enduring alterations in neurobehavioral responses associated with early life manipulations reflect changes in maternal behavior. Thus, we sought to determine if neonatal isolation alters maternal care and if dams with neonatal isolation experience as pups showed differential maternal care towards their pups. In Experiment 1, litters were assigned to one of three conditions: neonatal isolation, handled (5-min separation of dam from litter), or non-handled (no separation). Maternal behaviors were rated on PND 2-9 for 60-min immediately following reunion of mother and litter. In Experiment 2, female rats with or without neonatal isolation experience were assigned to either the neonatal isolation or non-handled litter condition and maternal behaviors rated. Dams of isolated and handled litters spent more time licking pups and less time picking up pups to put outside the nest than dams of non-handled litters. Further, dams of isolated and handled vs non-handled litters showed less non-maternal behaviors of burrowing and grooming. Neonatal isolation-experienced dams with isolated litters failed to increase pup-licking and decrease non-maternal behaviors. Rather, these dams picked up pups to place outside the nest more than non-handled-experienced dams. Neonatal isolation alters maternal behavior that, in turn, may shape neurobehavioral responses of offspring including effects on maternal care. Such changes may reflect epigenetic effects resulting from changes in maternal behavior.

Keywords

neonatal handling; early life stress; mother-pup behavior; pup licking

1. Introduction

Rats are altricial animals and require large investments of parental care for several days after birth (Rosenblatt and Snowdon, 1996). The repertoire of maternal behaviors is under neurohormonal and sensory controls (Stern, 1989). Pups use olfactory, visual, and auditory

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cues to signal the dam when separated from her (Brewster and Leon, 1980; Smotherman et al., 1974; Stern, 1989). These signals induce the dam to retrieve her pups into a nest after which she hovers over and licks them usually in the anogenital area. Pups are active participants in the licking interaction. Indeed, the mother-infant relationship is considered symbiotic because it is beneficial for the dam and the pups (Gubernick and Alberts, 1983, 1987). Both the dam and pups are actively involved in the nursing that usually occurs after pup-licking (Stern and Johnson, 1989, 1990). The degree and types of maternal behavior expressed vary among individuals and across strains (Gomez-Serrano et al., 2002; Liu et al., 1997; Moore et al., 1997; Myers et al., 1989). Such individual differences in maternal behavior can be transmitted across generations (Boccia and Pedersen, 2001; Francis et al., 1999).

The neural circuitry of maternal behaviors is complex and not fully understood (Gammie, 2005; Numan and Sheehan, 1997; Stern and Lonstein, 2001). Yet, a key neural component that contributes to maternal behavior is the mesolimbic dopamine (DA) system including the DA-containing cell bodies of the ventral tegmental area (VTA) and one of its projection sites, the nucleus accumbens (NAc). Administration of DA receptor antagonists into NAc of the dam impairs licking and pup retrieval behaviors (Keer and Stern, 1999; Numan et al., 2005) and 6-OHDA lesions of the VTA or NAc disrupt pup retrieval behavior (Hansen et al., 1991). Enhanced c-fos expression in NAc is seen in lactating dams upon pup presentation (Fleming et al., 1994) and dams show increased extracellular DA levels in NAc after reunion with a litter from which they were separated overnight (Hansen, 1994). Finally, variations in NAc DA signals are correlated with the degree and length of pup-licking bouts (Champagne et al., 2004). Behaviors of the pups that induce maternal care appear to involve DAergic mechanisms as well. For example, striatal 6-OHDA lesions in pups increases pup retrieval and nest-building times and decreases huddling times of the dam (Wilkins et al., 1997). Further, DAergic agents administered to pups alter isolation-induced increases in ultrasonic vocalizations (Kehoe and Boylan, 1992; Muller et al., 2009). Finally, molecular genetic manipulations in mouse that target DA system alter maternal behavior (Spielewoy et al., 2000). Thus, like other motivated behaviors (Robbins and Everitt, 1996), DA in the NAc of either the pup or the dam makes an important contribution to motivated pup and maternal behaviors.

DA in NAc has long been associated with drug reward (Koob and Bloom, 1988; Wise and Rompre, 1989). We find that neonatal isolation experienced as a pup facilitates the increase in ventral striatal extracellular DA levels in response to cocaine administration in both infant and adult rats (Kosten et al., 2003; Kosten et al., 2005b). Further, adult rats with neonatal isolation experience show enhanced cocaine self-administration (Kosten et al., 2000; Kosten et al., 2004a; Kosten et al., 2006; Zhang et al., 2005). In neonatal isolation, pups are individually isolated from dam, nest, and siblings for 1-hr a day on Postnatal Days (PND) 2-9. In addition to alterations in ventral striatal DA system and on behavioral responses to psychostimulants, neonatal isolation also alters corticosterone levels basally and in response to stress (Kosten et al., 2000; McCormick et al., 1998; McCormick et al., 2002),

Similar to neonatal isolation, other early life manipulations, such as handling, the brief (e.g., 5-min) removal of the dam from the rat pups and nest, or maternal separation, a more long-term (e.g., >3-hr) removal, have long-term effects on hypothalamic-pituitary-adrenal (HPA) axis function and on various behaviors (Ader and Grotta, 1969; Anisman et al., 1998; Hess et al., 1969; Kehoe and Shoemaker, 2001; Lehmann and Feldon, 2000; Levine, 1957; Meaney et al., 1996) including drug self-administration (Jaworski et al., 2005; Moffett et al., 2006). Handling and maternal separation also affect maternal care (Barnett and Urn, 1967; Bell et al., 1971; Hofer, 1983; Lee and Williams, 1974; Levine, 1987; Liu et al., 1997; Marmendal et al., 2004; Pryce et al., 2001). Differences in maternal behaviors have been related to HPA axis functioning in the adult offspring (Liu et al., 1997). The changes in maternal behavior induced by maternal separation and experienced by the pup are associated with acquisition of cocaine

and ethanol self-administration in adulthood (Francis and Kuhar, 2008). Specifically, the less licking received as a pup correlates with an increased ease of acquisition of drug self-administration.

Given that neonatal isolation alters neurochemical and behavioral responses to cocaine as well as affects the HPA axis system, it likely modifies maternal behavior. Pups with neonatal isolation experience may receive dissimilar amounts of licking and retrieval that, in turn, may affect neurohormonal systems involved in the behavioral differences seen later in life. Further, neonatal isolation may alter the expression of maternal behavior in adulthood as shown previously in female rats with handling, maternal deprivation, or artificial rearing experiences (Boccia and Pedersen, 2001; Francis et al., 1999; Gonzalez et al., 2001; Lovic et al., 2001). Thus, the first experiment sought to determine whether neonatal isolation alters maternal behavior post-reunion of dam and pups. Maternal behaviors may change across repeated isolations perhaps leading to enhanced effects. Indeed, our previous research shows that corticosterone levels of pups increase from the first to the last isolation (McCormick et al., 1998). A prior study of neonatal social isolation reports that the emission of ultrasonic vocalizations by pups decrease with repeated isolations (Zimmerberg et al., 2003). Thus, we examined mother-pup interactions across the 8-days of neonatal isolation and compared them to litters that were handled (brief removal of litter from nest) or were non-handled. The second experiment assessed whether female rats with neonatal isolation experience as pups show altered maternal behavior towards their pups in response to having their litters isolated. We also assessed fertility (percent successful pregnancies) and fecundity (number of pups/litter) in neonatally isolated dams vs. non-handled dams. Although we found no effect of neonatal isolation on the estrous cycling (Kosten et al., 2005a) consistent with a prior study of maternal separation (Rhees et al., 2001), maternal behavior has been shown to modulate female reproductive function (Cameron et al., 2008; Uriarte et al., 2007).

2. Materials and Methods

2.1 General Methods

2.11 Subjects and housing—Male and female Sprague-Dawley rats (Charles River, MA) were housed in polypropylene cages in a temperature- and humidity-controlled colony room maintained on a 12:12 light/dark cycle (lights on at 0700). Food and water were available *ad libitum*. Sets of one male and two females were paired for breeding. Pregnant females were then transferred to individual cages. Litters found before 1700 hr were considered born on that day (Postnatal Day 0). On Postnatal Day 1 (PND1), litters were culled to 12 pups, 6 males and 6 females, whenever possible. Pups that were to be studied in adulthood (Experiment 2) were weaned on PND25 and caged in pairs of same sex, same treatment condition in the colony room. All procedures were approved by the institutional Animal Care and Use Committees in accordance with guidelines set forth by NIH.

2.12 Neonatal treatment conditions—Some litters were assigned to the neonatal isolation condition and this procedure was described previously (Kehoe and Shoemaker, 2001; Kosten and Kehoe, 2005). Briefly, starting on PND2, each pup was weighed and placed individually in an opaque plastic container (9-cm diameter and 8-cm deep) with no bedding for 1 hr/day (between 0900 and 1200) in a heated (30° C), humidity-controlled chamber with white noise to mask other pups' calls. Containers were placed 20–30 cm apart. This isolation procedure was performed daily through PND9 in a test room separated from the main laboratory and colony rooms. Litters in the handled group were removed as a group from the dam and cage, weighed, and then returned to the cage spaced around the dam. This separation time varied between 5 and 10-min which is within the timeframe defined as handling. Non-handled rats originated from litters born in the colony about the same time and were culled to 12 pups (6

males and 6 females when possible) on PND1, but were not handled or disturbed on PND2-PND9 except for the videotaping procedure.

2.13 Videotaping and behavioral scoring procedure—Dams were transported in their home cage to a second test room for videotaping approximately 1-hr before cessation of the isolation or handling of the litters or, in the case of the non-handled litters, were transported with their litters. After the transport, litters were isolated, handled, or not handled. When it was time to replace the pups, the wire top of the cage was replaced with a clear, Plexiglas top to enhance viewing. The cage was placed on a table located below a video camera (Panasonic) suspended from the ceiling and connected to a DVC camcorder (Panasonic). Pups from the isolate and handled litters were returned to the dam and spaced around the cage. After placement of all the pups, the videotaping session began. Dams and their litters were returned to the colony room following these sessions. Only one litter was videotaped at one time. Videotapes were rated at a later date by an observer who was not informed of the litter or dam treatments. The amount of time (s) spent in each behavior was recorded during each of twelve 5-min intervals (total session time = 60-min). Several behaviors were measured and classified as either pup-directed (e.g., licking) or nonpup-directed (e.g., burrowing). These variables are listed and defined in Table 1.

2.2 Experiment 1: Immediate effects of neonatal isolation and handling on maternal behaviors

2.21 Groups—Litters were assigned to one of the following three treatment conditions in a random manner: 1) isolate; 2) handled; and 3) non-handled. The neonatal treatment conditions were described above. There were 8 litters in each of these three treatment conditions. Parity did not differ between groups and each group included dams that were experienced or primiparous. Moreover, parity was not associated significantly with any behavioral measure and thus data were collapsed across this dimension.

2.22 Videotaping procedure—Videotaped sessions were 60-min in length and were performed daily on PND2-PND9 (between 0900 to 1200 hr).

2.23 Data analysis—Data on times spent in each behavior during each 5-min interval were averaged (mean) across two- or three-day blocks (PND2-PND4; PND5-PND7; PND8-PND9). This resulted in three data points per variable representing the factor of day. These data were analyzed using $3 \times 3 \times 12$ ANOVA representing the between group factor of litter treatment (isolate, handled, non-handled) with repeated measures on day and time. Data on total time creating a huddle were analyzed using 3×3 ANOVA representing the between group factor of treatment group with repeated measures on day. The Greenhouse-Geiser adjustment was employed for repeated measures effects.

2.3 Experiment 2: Enduring effects of neonatal isolation on maternal behaviors

2.31 Groups—Adult female rats that had neonatal isolation experience as described above or were non-handled as pups were mated with male breeders (i.e., male rats that had not been assigned to any specific early manipulation). Each female was obtained from subjects used in Experiment 1 and assigned to one of two litter treatment conditions: 1) isolate or 2) non-handled. This resulted in four groups: 1) isolate dams with isolate litters; 2) isolate dams with non-handled litters; 3) non-handled dams with isolate litters; 4) non-handled dams with non-handled litters. There were 10–11 litters in each group and all dams were primiparous.

2.32 Videotaping procedure—Videotaped sessions were conducted on PND2, PND5, and PND9. For isolate litter groups, the isolation procedure was performed on intervening days as

before. Sessions were reduced to 30-min in length based on results of Experiment 1 in which minimal behaviors were expressed after this time point.

2.33 Data analyses—Data on times spent in each behavior during each 5-min interval were analyzed with $2 \times 2 \times 3 \times 6$ ANOVA. This represents the between group factors of dam treatment (isolate, non-handled) and litter treatment (isolate, non-handled) with repeated measures on day and time blocks. The Greenhouse-Geiser adjustment was employed for repeated measures effects. Two variables, latency to first retrieval and latency to create a huddle, were analyzed with 2-way (dam treatment) Kruskal-Wallis ANOVA. This was necessary because some dams failed to retrieve pups or create a huddle before the allotted time was over. Non-parametric statistics allowed inclusion of these data.

3. Results

3.1 Experiment 1: Immediate effects of neonatal isolation and handling on maternal behaviors

3.11 Pup-directed behaviors—The total times spent creating a huddle are presented in Fig. 1 by group. Times spent creating a huddle differ significantly by Group, $F(2,20)=7.43$; $P<0.005$, but do not differ across days ($P>0.10$). As seen in Fig. 1, dams of litters of isolate and handled pups spend significantly more time creating a huddle compared to dams of litters of non-handled pups. This is supported by significant comparisons of dams of isolate vs non-handled litters, $F(1,14)=10.61$; $P<0.01$ and of dams of handled vs. non-handled litters, $F(1,13)=25.10$; $P<0.005$. There is a trend for dams of isolate litters to spend more time creating a huddle compared to dams of handled litters, $F(1,13)=3.45$; $P<0.09$. The main effect of day and its interaction terms are not significant (P 's >0.10).

The times spent picking up pups and placing them outside the nest are presented in Table 2 by group and day. As seen in Table 2, dams of non-handled litters spend more time picking up pups to place outside the nest compared to dams of handled litters which, in turn, spend more time picking up pups to place outside the nest compared to dams of isolate litters. This statement is supported by the significant Group effect, $F(2,21)=13.58$; $P<0.0001$, and by the significant comparisons of dams with non-handled vs. isolate litters, $F(1,11)=26.39$; $P<0.0001$, dams with non-handled vs. handled litters, $F(1,11)=7.44$; $P<0.05$, and dams with handled vs. isolate litters, $F(1,11)=6.37$; $P<0.02$. There are no significant Day or Time effects nor interactions with Group (P 's >0.10).

The times spent licking pups are shown in Fig. 2 by time, day, and group. As seen in Fig. 2, dams of isolate and handled litters spend more time licking their pups compared to dams of non-handled litters. This is supported by the significant Group effect, $F(2,21)=6.49$; $P<0.005$ and by the comparisons of dams of isolate vs. non-handled litters, $F(1,11)=9.23$; $P<0.01$ and dams of handled vs. non-handled litters, $F(1,11)=10.38$; $P<0.01$. Times spent licking pups increase during the initial time periods over days particularly for dams of isolate and handled pups. This statement is supported by the significant effects of Day, $F(2,42)=13.91$; $P<0.0001$, Time, $F(11,231)=7.38$; $P<0.0001$, Day $\times$ Time, $F(22,462)=2.83$; $P<0.001$, and Group $\times$ Time, $F(22,231)=1.88$; $P<0.05$.

3.12 Nonpup-directed behaviors—The times spent in various nonpup-directed behaviors are presented in Fig. 3 by day and group. The times the dams spent grooming themselves is greater for those of handled and non-handled litters compared to dams of isolate litters. This statement is supported by the significant Group effect, $F(2,21)=3.98$; $P<0.05$ and by the significant comparisons of dams of handled vs. isolate litters, $F(1,11)=6.74$; $P<0.05$; and dams of non-handled vs. isolate litters, $F(1,11)=5.11$; $P=0.05$. The times the dams spent burrowing the bedding of the cage differed by group as seen in Fig. 3. Dams of non-handled litters burrow

more than dams of handled or isolate litters. This statement is supported by the significant Group effect, $F(2,21)=9.48$; $P<0.0005$ and by the significant comparisons of dams of non-handled vs. handled litters, $F(1,11)=14.38$; $P<0.001$; and dams of non-handled vs. isolate litters, $F(1,11)=10.79$; $P<0.005$. The times spent climbing differed by group as seen in Fig. 3. Dams of handled litters climb more than dams of non-handled or isolate litters. This statement is supported by the significant Group effect, $F(2,17)=16.41$; $P<0.0001$; and by the significant comparisons of dams of handled vs non-handled litters, $F(1,11)=38.59$; $P<0.0001$; and dams of handled vs. isolate litters, $F(1,11)=12.21$; $P<0.001$. The main effect of day and its interaction terms are not significant ($P's>0.10$).

3.2 Experiment 2: Enduring effects of neonatal isolation on maternal behaviors

3.21 Pregnancy and birth factors—The pregnancy and birth factors for neonatally isolated dams do not differ from the non-handled dams as seen in Table 3. There are no group differences in age or body weight prior to mating ($P's>0.10$) as seen in Table 3. The fertility and fecundity factors of successful mating and number of pups in the litter do not differ by group ($P's>0.10$).

3.22 Pup-directed behaviors—Latencies to first pup retrieval and huddle creation are shown in Table 4. These variables could only be assessed in dams of isolate litters because 70% of dams of non-handled litters failed to initiate creating a huddle. There are no differences between dams that were isolated as pups and dams that were non-handled as pups in either measure nor do these measures change significantly over days ($P's>0.10$).

The times dams spend licking their pups collapsed across test sessions are shown in Fig. 4. Dams that were non-handled as pups and have isolate litters tend to lick their pups more than the other groups as supported by the trend towards significance of the Dam treatment $\times$ Litter treatment interaction, $F(1,37)=3.85$; $P<0.06$. The main effects of Dam treatment and Litter treatment are not significant nor are any of the interactions with Day ($P's>0.10$). The times spent picking up pups and placing outside the nest are greatest in dams that were isolated as pups and have isolate litters as seen in Fig. 4. These data are collapsed across test sessions. These statements are supported by the significant Dam Treatment $\times$ Litter Treatment, $F(1,37)=5.05$; $P<0.05$, and Day $\times$ Dam Treatment $\times$ Litter Treatment, $F(1,37)=4.22$; $P<0.05$, interactions. The main effects of Dam and Litter treatments are not significant ($P's>0.10$).

3.23 Nonpup-directed behaviors—There are no significant Dam or Litter treatment effects on nonpup-directed behaviors of self-grooming or climbing (data not shown). However, the nonpup-directed behavior of burrowing showed a significant Dam Treatment $\times$ Litter Treatment, $F(1,37)=6.40$; $P<0.05$. As shown in Fig. 5, dams that were non-handled as pups exhibited much less burrowing behavior if their litter was isolated vs. when their litter was non-handled. In contrast, litter treatment had little effect on burrowing behavior in dams that were isolated as pups. The main effects of Dam Treatment and Litter treatment failed to reach significance for this behavior ($P's>0.10$).

4. Discussion

The results of this study show that neonatal isolation and handling alter maternal behavior immediately post-reunion. Dams of isolate and handled litters spend more time creating a huddle and licking pups as well as less time picking up pups to place outside the nest compared to dams of non-handled litters as seen in Experiment 1. Further, the times spent licking pups increase earlier in the sessions over days in the neonatal isolation and handled groups. There are also effects of the neonatal treatment conditions on nonpup-directed behaviors. All three of these behaviors, self-grooming, burrowing, and climbing, are expressed the least by dams

of isolated litters. Compared to dams of isolated litters, dams of non-handled litters spend more time grooming and burrowing and dams of handled litters spend more time grooming and climbing. Data obtained from Experiment 2 largely confirm these findings. Control, non-handled dams with isolated litters tend to lick their pups more than the other groups. These dams also show a decrease in the nonpup-directed behavior of burrowing compared to dams of non-handled litters. In contrast, dams that experienced neonatal isolation as pups do not show a decrease in burrowing nor an increase in pup licking in response to having their litter isolated. Further, such dams spend more time picking up these pups to place outside the nest in contrast to results from Experiment 1 in which dams of isolated litters expressed this behavior the least. Thus, the neonatal treatments of handling and neonatal isolation appear to disrupt the presumed typical maternal behaviors as assessed by comparisons to the non-handled condition. In addition to this immediate effect, neonatal isolation has enduring effects on maternal behavior exhibited in adulthood.

Maternal behavior occurs in bouts (Grota and Ader, 1969) and the disruptions of pup removal for handling or neonatal isolation appear to have stimulated a bout of behavior. The behaviors expressed follow a pattern in which the dam initially spends large amounts of time retrieving pups into a huddle and then licking them. Over days, the timing or pattern of these behaviors changes. Latencies to initiate and create a huddle tend to increase and licking occurs earlier. These changes likely reflect, to some extent, the maturation of the pup and the resulting alteration in maternal behavior (Grota and Ader, 1969; Stern and MacKinnon, 1978). However, the changes may also be in response to the repeated manipulation of removal from the pups. Cues associated with the neonatal treatment manipulation and the videotaping sessions are similar across days. The dams and pups may have come to anticipate these manipulations and this could affect the timing and degree of the behaviors expressed.

Assessments of maternal behavior were made at multiple time points in this study because we were interested in whether sensitization, or an increase in behavioral responses, would occur. This hypothesis was based on our prior findings that isolation-induced increases in plasma corticosterone levels were greater in 10-day old pups that had 9 days of isolation compared to pups without this prior experience (McCormick et al., 1998) and that cross-sensitization to amphetamine is also seen (Kehoe et al., 1998). To a large extent, we failed to find evidence of sensitization in the present study. In Experiment 1, the only behavior that changed over days was pup licking and this decreased over days. There was one behavior in Experiment 2 that showed a significant interaction with the factor of Day, picking up pups to place outside the nest. However, this interaction mainly reflected that dams with isolation experience that had their litters isolated showed a slight increase in this behavior on the second and third test sessions whereas the dams with non-handling experience that had non-handled litters showed a slight decrease in this behavior over test days. The other two groups spent very little time picking up their pups to place outside the nest across the three test sessions. A prior study examining the effects of neonatal social isolation reports decreases in ultrasonic vocalizations by the pups across days (Zimmerberg et al., 2003). Our procedure differs somewhat from that of Zimmerberg and we did not measure pup vocalizations. Nonetheless, it is possible that the relative lack of changes in maternal behavior over days seen in the present study reflects that decreases in the pup's signaling behaviors counteracted potential increases in the mother's behavior.

A significant question addressed in this study was whether having neonatal isolation experience as a pup changes maternal behavior expressed in adulthood. Maternal behavior seen in Experiment 1 in response to having litters isolated or non-handled was used as a baseline. In addition, dams with neonatal isolation were compared to dams without this experience in Experiment 2. The most striking effect on the dam of having neonatal isolation experience as a pup is that it increases the behavior of picking up the pup and placing it outside the nest in

response to having her litter isolated. Dams without neonatal isolation experience exhibit low levels of this behavior when their litters have been isolated as compared to dams with litters that were non-handled as seen in Experiment 1. It is difficult to explain this dramatic effect of neonatal isolation on maternal behavior. There were no differences in latencies to begin pup retrieval or in times to create a huddle between dams with and without neonatal isolation experience. Yet, in Experiment 1, dams with isolated litters or handled litters spent more time creating a huddle. This may reflect some inefficiency or disorganization in the pup retrieval process although isolation of litters decreased the times spent picking up the pup to put outside the nest in Experiment 1. Pup retrieval and picking up pups to put outside the nest may be opposing behavioral tendencies.

Prior research has shown that the early life manipulations of handling and maternal separation affect mother-pup interactions (Barnett and Urn, 1967; Bell et al., 1971; Hofer, 1983; Lee and Williams, 1974; Levine, 1987; Liu et al., 1997; Marmendal et al., 2004; Pryce et al., 2001). The results of the present study add to that literature by demonstrating that neonatal isolation also alters these behaviors. As seen previously, a manipulation that removes pups from the dam leads to a bout of increased pup-licking upon reunion of litter and dam (Boccia and Pedersen, 2001; Lee and Williams, 1974; Liu et al., 1997; Marmendal et al., 2004; Pryce et al., 2001; Zimmerberg et al., 2003). This effect was confirmed in the present study with the handled litter group and extended to the manipulation of neonatal isolation. More prolonged maternal separations either decreased pup licking compared to pups subjected to brief separations when assessed almost 3-hr post-reunion (Boccia and Pedersen, 2001) or stimulated a bout of increased pup licking immediately post-reunion but not at other times (Pryce et al., 2001). Yet, there are differences across these early life manipulations. For example, handling and maternal separation typically involve the removal of the dam from the litter but the pups remain together in a huddle. This allows the pups to maintain body temperature and receive tactile stimulation. In contrast, neonatal isolation involves both removal of the dam and individual isolation of pups. The neonatal social isolation procedure used by Zimmerberg et al (2003) differs from the present procedure because in her study some pups from the litter are isolated while other pups remain with the dam. Thus, handling and maternal separation may be stressful to the dam and neonatal social isolation may be stressful to the pups and our neonatal isolation procedure may be stressful to both. In addition to these procedural differences, the isolation/separation times differ across manipulations with handling the shortest, maternal separation the longest, and neonatal isolation intermediate. And the post-natal days on which these manipulations are performed vary across studies.

The duration of isolation or separation (handled groups) received postnatally appears to be related to its effect on maternal behaviors. In some cases, neonatal isolation, that is 1-hr in length, has greater effects than handling that is 5–10 min in length. There is a trend for dams of isolated litters to spend more time creating a huddle compared to dams of handled litters. Dams of isolated litters spend less time picking up pups to place outside the nest compared to dams of handled litters. And, dams of isolated litters show a shift to increased time licking their pups early in the session by Days 5–7, whereas dams of handled litters exhibit this shift to lick pups earlier in the session on the last days of the procedure. Finally, times spent in two of the three nonpup-directed behaviors (grooming and climbing) are greater in dams of handled litters vs. dams of isolated litters. Perhaps compared to 5–10 min of handling, pups that were isolated for 1-hr produce greater or more prolonged stimuli that induce maternal behavior (e.g., ultrasonic vocalizations) and/or the dams of these pups have experienced a greater stressor.

Pup retrieval behavior is also stimulated in the dam by separation from her litter. In fact, separation from the litter compensates for deficits in pup retrieval behavior caused by 6-OHDA lesions of the VTA or NAc (Hansen, 1994). This suggests that regions besides the VTA-NAc DA pathway contribute to pup retrieval. One such area is the lateral septum (Fleischer and

Slotnick, 1978). Lateral septal-lesioned dams pick up their pups after they are scattered about the cage but they carry them around and put them in places outside the nest. A similar behavior is seen in Experiment 2 in which dams that experienced neonatal isolation as pups pick up their pups that were isolated and put them outside the nest. It is possible that this reflects effects on the lateral septum as we previously showed altered DA turnover in lateral septum of pups immediately following nine days of neonatal isolation (Kehoe et al., 1998). Whether such alterations endure into adulthood is unknown. Some isolation-induced neurochemical changes do endure into adulthood while others do not (Kosten et al., 2003, 2004b; Kosten et al., 2005b).

Previously, we have demonstrated that neonatal isolation has immediate and enduring effects on neurochemical responses in ventral striatum to psychostimulant administration (Kehoe et al., 1996a; Kehoe et al., 1996b; Kosten et al., 2003; Kosten et al., 2005b) and that it facilitates cocaine self-administration in adulthood (Kosten et al., 2000; Kosten et al., 2004a; Kosten et al., 2006; Zhang et al., 2005). The present study extends our research findings on immediate and enduring effects of neonatal isolation to maternal behavior. It is possible that the changes in mother-pup behaviors reflect the documented neonatal isolation induced changes in the mesolimbic DA system in either the pup or the dam or both, as this is an important component of the neural circuitry of maternal behavior (Gammie, 2005; Numan and Sheehan, 1997; Stern and Lonstein, 2001). Neonatal isolation does not compromise fertility or fecundity when the dams are adults but it does lead to some disruptions in maternal care. The lack of effect of neonatal isolation on reproductive function is also suggested by our prior finding of no change in estrous cycling (Kosten et al., 2005a). Maternal separation has also been found to not alter estrous cycling (Rhees et al., 2001) although reproductive function of females is modulated by maternal care (Cameron et al., 2008; Uriarte et al., 2007).

The mesolimbic DA system also contributes to drug reward (Koob and Bloom, 1988; Robbins and Everitt, 1996; Wise and Rompre, 1989) and specifically, to drug self-administration behavior (Bergman et al., 1989; Ritz et al., 1987). Drug self-administration and maternal behavior received have been linked; cocaine and ethanol self-administration in adulthood is associated with lower levels of licking received as a pup (Francis and Kuhar, 2008). Our data suggest the opposite relationship because neonatal isolation increased licking received as a pup in the present study and our prior work shows it also facilitates cocaine self-administration (Kosten et al., 2000; Kosten et al., 2004a; Kosten et al., 2006; Zhang et al., 2005). Methodological differences may explain the discrepancy. In contrast to the present study in which maternal behavior was rated immediately following isolation, Francis and Kuhar (2008) examined these behaviors beginning at least 1-hr after cessation of maternal separation. And, as described above, maternal separation procedures has many parametric differences from neonatal isolation. Thus, there are clear fundamental differences between the effects of maternal separation vs. neonatal isolation on drug self-administration in adulthood for which the underlying mechanisms are not known.

Emerging data suggest that maternal behavioral patterns transmit across generations (Francis et al., 1999; Gonzalez et al., 2001; Lovic et al., 2001). Francis et al. (1999) report that the amount of time a dam spends in maternal care correlates with the time her offspring will spend in maternal care as an adult. Gonzalez et al. (2001) report that dams that were reared artificially (i.e., were not licked as pups) showed less licking and crouching behaviors when caring for their own pups. The results from the present study are not consistent with this notion. However, other research (Boccia and Pedersen, 2001; Pryce et al., 2001) would suggest that neonatal isolation alters maternal care at other times and in ways that differ from the immediate post-reunion observational period examined in the present study. Future studies should examine mother-pup behaviors at other times in addition to the immediate post-reunion period. Further, the enduring effects of neonatal isolation may result from epigenetic changes induced by

alterations in maternal behavior. Previous work demonstrates that licking and grooming of the pup affects the expression of a glucocorticoid gene via DNA methylation and histone deacetylation and such effects are related to the stress hormone phenotype of the adult (Liu et al., 1997; Szyf et al., 2005; Weaver et al., 2004). The possibility that epigenetic effects occur in some as yet unknown gene or set of genes due to neonatal isolation-induced changes in maternal behavior needs to be explored to better understand the mechanisms that contribute to the immediate and enduring effects of this early life manipulation.

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Figure 1.

Mean $\pm$ S.E.M. time (s) spent creating a huddle during 60-min test sessions performed immediately post-reunion are shown for dams of isolate (ISO; open squares), handled (HAND; closed circles), and non-handled (NH; closed squares) litters by post-natal day. Points reflect the mean of the two or three day blocks (PND 2-4; PND 5-7; PND 8-9). Dams of isolate and handled litters spend significantly more time creating huddles than dams of non-handled litters.

Figure 2.

Mean $\pm$ S.E.M. time (s) spent licking pups by 5-min time blocks during the first 30-min of the 60-min test sessions performed immediately post-reunion are shown for dams of isolate (ISO; open squares), handled (HAND; closed circles), and non-handled (NH; closed squares) litters. Means for the first three post-natal (PND) days (PND 2-4) are shown in the top panel. Means for PND 5-7 are shown in the middle panel and means for PND 8-9 are shown in the bottom panel. Dams of isolate and handled litters spend significantly more time licking pups than dams of non-handled litters and this effect occurs earlier in the session over days.

Figure 3.

Mean $\pm$ S.E.M. time (s) spent self-grooming (top panel), burrowing (middle panel) and climbing (bottom panel) across days are presented for dams of isolate (ISO; open squares), handled (HAND; closed circles), and non-handled (NH; closed squares) litters. Times spent in these nonpup-directed behaviors differed by treatment group.

Figure 4.

Mean (+ S.E.M.) times (sec) spent licking pups (top) and picking up pups to the place outside the nest (bottom) by dams that were isolated or non-handled as pups and have non-handled (NH; closed bars) or isolate (ISO; open bars) litters. Data are averaged across postnatal test days. Dams that were non-handled as pups tend to spend more time licking pups that were isolated. Dams that were isolated as pups spend more time picking up isolated pups to place outside the nest compared to the other groups.

Figure 5.

Mean (+ S.E.M.) times (sec) spent burrowing by dams that were isolated or non-handled as pups and have non-handled (NH; closed bars) or isolate (ISO; open bars) litters. Data are averaged across postnatal test days. Dams that were isolated as pups and have isolated litters spend more time burrowing.

Table 1

Definitions of maternal behaviors. All measures of time are in seconds.

Variable	Definition
<i>Study 1</i>	<i>Pup-directed behaviors</i>
Huddle creation	Time dam spends picking up pups to move to a huddle in the cage.
Pick-up	Time dam spends picking up pups and placing them in a location in the cage other than the nest.
Licking	Time dam spends licking any part of a pup's body.
<i>Study 1</i>	<i>Nonpup-directed behaviors</i>
Climbing	Time dam places one or both paws on the wall of the cage.
Burrowing	Time dam engages in displacing the cage bedding with her nose or paws.
Grooming	Time dam spends licking or stroking herself.
<i>Study 2</i>	<i>Pup-directed behaviors</i>
Latency to 1 st retrieval	Time before dam picks up a pup and moves it to another location in cage.
Latency to create huddle	Time before all pups are huddled in physical contact with one another.
Pick-up	See Study 1 above.
Licking	See Study 1 above.

Table 2

Mean ($\pm$ S.E.M.) total times (s) spent picking up pups and placing in a location other than the nest over 60-min sessions in isolate, handled, and non-handled litters (n=8/condition) over 3 blocks of days from PND2-PND9.

Day *	Isolate	Handled	Non-handled
PND 2-4	4.1 (3.4)	8.8 (3.6)	13.1 (4.8)
PND 5-7	2.1 (1.6)	2.6 (1.4)	16.5 (6.9)
PND 8-9	1.0 (0.7)	6.2 (5.4)	6.9 (4.6)

* Significant Litter Treatment effect

Table 3

Mean ($\pm$ S.E.M.) pregnancy factors and percentage of successful matings for isolate and non-handled dams.

Variable	Isolate Dams	Non-handled Dams
Dam age (days)	97.4 (3.3)	97.2 (0.8)
Dam body weight (g)	244.7 (10.0)	254.0 (6.7)
% successful matings	86	84
# pups in litter	14.1 (0.5)	14.2 (0.6)

Table 4

Median ($\pm$ semi-interquartile range) latency (s) to initiate and total time (s) spent creating a huddle after neonatal isolation of pups in dams that were isolated or non-handled as pups across postnatal days.

Variable	Isolate Dams			Non-handled Dams		
	PND 2	PND 5	PND 9	PND 2	PND 5	PND 9
Latency	25 $\pm$ 49	95 $\pm$ 138	107 $\pm$ 70	47 $\pm$ 49	47 $\pm$ 33	43 $\pm$ 29
Complete	449 $\pm$ 218	396 $\pm$ 276	692 $\pm$ 177	289.5 $\pm$ 179	371 $\pm$ 217	382 $\pm$ 295

Table 5

Mean ($\pm$ S.E.M.) times (s) spent licking pups and picking up pups to the place outside the nest (Pick-up) by dams that were isolated or non-handled as pups and have non-handled (NH) or isolate litters. Data are averaged across postnatal test days.

Litter	Isolate Dams		Non-handled Dams	
	NH litter	Isolate litter	NH litter	Isolate litter
Licking *	161.7 (34.3)	190.3 (34.0)	204.1 (32.0)	247.5 (31.0)
Pick-up **	2.9 (1.3)	11.9 (6.6)	5.4 (3.1)	3.5 (1.5)

* Dam treatment $\times$ Litter treatment: $P < 0.06$

** Dam treatment $\times$ Litter treatment: $P < 0.05$

Table 6

Mean ($\pm$ S.E.M.) times (s) spent burrowing by dams that were isolated or non-handled as pups and have non-handled (NH) or isolate litters. Data are averaged across postnatal test days.

Litter	Isolate Dams		Non-handled Dams	
	NH litter	Isolate litter	NH litter	Isolate litter
Burrow **	174.9 (59.7)	271.3 (108.3)	187.1 (61.3)	60.6 (20.5)

** Dam treatment $\times$ Litter treatment: $P < 0.05$