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Long-term Behavioral Consequences of Brief, Repeated Neonatal Isolation

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Abstract

Rats subjected to stressful stimuli during the stress hyporesponsive period exhibit varied neuroendocrine and behavioral changes as neonates, adolescents and adults. The current work examined the effects of neonatal isolation stress, using a within-litter design, on adult anxiety-related behavior and endocrine stress reactivity. Neonatal rats were isolated daily for 1 hr from postnatal day (P) 4-9, a manipulation previously shown to induce hypothalamic-pituitary-adrenal (HPA) responses on P9 (Knuth and Etgen, 2005). Control animals were either handled briefly or left undisturbed (with-dam). Adult rats were tested for anxiety-related behavior using the elevated plus maze and open field, and for endocrine responses following restraint stress. Neonatal isolation decreased center exploration of the open field following 1 hr restraint, including decreased time in the center compared to with-dam or handled controls, and decreased center entries and distance traveled in the center compared to with-dam controls. It also decreased time in and entries into the open arms of the elevated plus maze compared to handled controls, suggesting enhanced anxiety-related behavior. Neonatal isolation had no effect on basal or restraint-induced levels of ACTH or corticosterone. These findings indicate that neonatal isolation may enhance anxiety-related behaviors, especially in response to stress, without altering HPA function.

Section: Cognitive and Behavioral Neuroscience

Keywords

neonatal isolation; elevated-plus maze; open field; ACTH; corticosterone

Introduction

Brief isolation of an individual pup from the dam and litter, when repeated for several days, is an effective method of stimulating the neonatal HPA axis without altering weight or growth in either neonates (McCormick et al., 1998; Huang et al., 2002; Knuth and Etgen, 2005) or adults (Kehoe et al., 1998; Kehoe and Bronzino, 1999). Daily 1 hr isolation from P2-8 significantly increased circulating corticosterone after 1 hr isolation on P9, whereas a single episode of isolation on P9 only did not, indicating that the adrenocortical response to a relatively mild stimulus emerged with repeated presentation (McCormick et al., 1998). When tested in adulthood, these animals display enhanced induction and maintenance of long-term potentiation in the medial perforant pathway (Kehoe and Bronzino, 1999), enhanced behavioral responses to restraint and amphetamine (Kehoe et al., 1998), and enhanced cocaine self-administration (Kosten et al., 2000). Recent studies suggest that both males (Kosten et al., 2005c; Zhang et al., 2005) and females (Kosten et al., 2004a; Kosten et al., 2005a; Kosten et al., 2005b) exhibit altered behavioral and neurochemical (Kosten et al., 2004b) responses to

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cocaine. Huang and colleagues (2002) report exacerbation of drug-induced seizure susceptibility, enhanced seizure-induced neuronal degeneration, and impairment of spatial memory in adults that were similarly stressed as neonates.

In our recent work, daily isolation took place from P4-8 so that the entire manipulation fell within the stress hyporesponsive period (SHRP). Only females subjected to daily isolation showed a significant increase in plasma corticosterone when isolated for 1 hr on P9. Conversely, both sexes showed a significant pituitary ACTH response on P9 (Knuth and Etgen, 2005). One significant finding of this work was that significant HPA responses were achieved using a stress paradigm that was comparatively more mild both in the total number and duration of episodes away from maternal care than previously reported. In the current work we take this model a step further and assess whether these comparatively brief episodes, which are significant enough to impact neonatal HPA responses, also have long-lasting implications for adult anxiety profiles. In particular, we are interested in whether brief, daily isolation has long-term effects on adult stress profiles similar to that reported for other, more protracted neonatal stress paradigms. To examine this question, we assessed anxiety-related behavior in the elevated plus maze and in the open field before and after a stressful challenge. While exposure to the novel arena represents a mild stressor for adult rats, early life stress is often without observable impact on activity levels unless a more potent stressor is imposed prior to activity measurements (Kehoe et al., 1995; Bronzino et al., 1996; 1996). Thus, a stressful challenge employed by others (1 hr restraint; (Kehoe et al., 1998; Estanislau and Morato, 2005)) was used prior to a second open field test. The endocrine stress response was assessed by measuring plasma ACTH and corticosterone before and after restraint stress in adult rats that were injected with DOI on P9 or that were with-dam, handled, or isolated as pups for 1 hr per day from P4-9.

Results

Body weight

Body weight was recorded prior to initiation of behavioral experiments to determine whether neonatal manipulations had any lasting impact on growth. Body weight did not differ among rats isolated (female = 229.6 ± 5.8 g, male = 344.3 ± 17.6 g), handled (female = 228.9 ± 5.6 g, male = 334.4 ± 17.6 g), or with-dam (female = 228.9 ± 5.6 g, male = 351.9 ± 9.3 g) as neonates. As expected, females weighed less than males ($p < 0.0001$).

Experiment I: Effects of neonatal isolation stress on adult stress reactivity Behavior in the elevated plus maze

Anxiety-like behavior was first measured using the EPM (Pellow *et al.*, 1985). There was no main effect of sex on arm entries or time spent in either closed or open arms of the EPM, nor did sex interact with any other factors. Therefore, data from both sexes were analyzed together. Rats isolated for 1 h daily from P4-8 spent a lower percentage of time in the open arms than handled rats ($p < 0.01$); however, with-dam and isolated rats did not differ significantly (Fig. 1a). Isolated rats spent significantly less time in the open arms (handled = 46.4 ± 11.0 sec, isolated = 31.0 ± 5.9 sec; $p < 0.05$) and more time in the closed arms (handled = 176.4 ± 14.9 sec, isolated = 219.8 ± 9.9 sec; $p < 0.05$) than handled rats. The proportion of entries isolated rats made into open arms was also significantly less than handled (Fig 1b; $p < 0.05$) but not with-dam rats. This is due to fewer open arm entries (handled = 4.8 ± 0.7 , isolated = 2.4 ± 0.4 ; $p < 0.01$) and equal closed arm entries (handled = 8.8 ± 0.7 , isolated = 7.6 ± 0.6) in these two groups. Handled rats also made a higher proportion of open arm entries than with-dam rats (Fig. 1b, $p < 0.05$).

Behavior in the novel open field

To determine whether neonatal isolation permanently altered adult motor function, which may influence performance on other behavior tests, general motor activity was determined over 10 min in the novel open field. No effect of neonatal isolation condition on distance traveled or velocity was evident upon first exposure to the open field (Table 1). There was a significant effect of sex; females traveled greater distance ($p<0.01$) and traveled faster ($p<0.01$) than males.

We examined exploratory and anxiety-related activity by quantifying each rat's rearing and time spent in the center of the open field. Adult rats did not differ on any measure of anxiety-related behavior (Table 1) in the novel open field, regardless of whether they had been isolated, handled, or with-dam as neonates. Females displayed less anxiety-related behavior than males, spending more time in the center of the open field ($p<0.05$) and making more crosses to ($p=0.01$) and traveling greater distance in ($p<0.01$) the center than males. Females also exhibited more exploratory activity (rears, $p<0.0001$) than male counterparts. Females also traveled through the center faster than males ($p<0.05$).

To examine whether rats habituated to the open field during the 10 min test, data from general motor and anxiety-related behavior were divided into two 5-min blocks and analyzed by repeated measures ANOVA. Decreased general motor activity in the arena and increased exploration of the center in the second 5-min epoch compared to the first were considered to be indicative of habituation. Rats traveled less distance ($p<0.0001$) and traveled more slowly in the second epoch ($p<0.001$). Anxiety-related behavior was also analyzed separately during the two 5-min epochs. Time in the center increased ($p<0.001$) and velocity through the center decreased ($p<0.001$) in the second time block compared to the first. There was no effect of time on crosses to or distance traveled in the center. Together, these data indicate that neonatally isolated and handled rats have normal motor activity and that they habituate normally to the open field.

Behavior in the open field after a stressful challenge

As with general motor activity prior to restraint challenge, females travel significantly more distance (Fig. 2a, $p<0.0001$) and faster (Fig. 2b, $p<0.0001$) than males. There was no significant effect of neonatal isolation on general motor activity, but there was a trend for neonatal isolation to decrease total distance traveled ($p=0.07$; Fig. 2a). When these data were analyzed in three 5-min blocks, similar results to the pre-restraint test were obtained, indicating this effect was not due to differential habituation among groups. Rats traveled less distance in each successive 5-min block ($p<0.0001$ for block 1 vs block 2; $p<0.01$ for block 2 vs. block 3), and they traveled slower between the first and second block ($p<0.0001$).

There was a significant main effect of neonatal stress on anxiety-related behavior in the open field after 1 hr restraint stress; this was true for time in the center ($F(1,46)=3.205$, $p<0.05$), crosses to the center ($F(1,46)=3.341$, $p<0.05$), and distance traveled in the center ($F(1,46)=4.167$, $p<0.05$). Rats isolated as neonates spent less time in the center than either handled or with-dam rats (Fig. 3a, $p<0.05$), made fewer crosses to the center (Fig. 3b, $p<0.05$) and traveled less distance in the center (Fig. 3c, $p<0.01$) than with-dam rats. There was a trend for neonatal stress to impact on rearing (Fig. 3d, $p=0.08$) as well as a main effect of sex ($F(1,46)=24.761$, $p<0.0001$) and a trend for interaction between the two ($p=0.07$). When females were analyzed separately, isolated females reared less than with-dam females ($p<0.05$) and tended to rear less than handled females ($p=0.06$). Finally, while females moved faster through the center than males following 1 hr restraint stress ($F(1,46)=21.993$, $p<0.0001$), previous neonatal stress had no impact on this measurement (data not shown). Females were more active than males, displaying more crosses through the center (Fig 3b), traveling more total distance in the center

(Fig 3c) and displaying more exploratory behavior (Fig 3d), although there was no effect of sex on time spent in the center (Fig 3a).

Similar to the novel open field condition, time in the center increased between the first and third 5-min block ($p < 0.01$), although velocity through the center was unchanged during the 15 min test. Crosses to the center also increased between the first and second 5-min block ($p < 0.05$). Distance traveled in the center and rearing did not change as a function of time. Neonatal group did not influence these measures, indicating that avoidance of the center observed in neonatally isolated adults is not due to altered habituation to the open field.

HPA activation

The effect of neonatal stress on adult HPA responsiveness was assessed by repeated blood sampling via jugular vein catheterization before and after 20 min restraint stress. Significant main effects for sex ($F(1,120)=23.365$, $p < 0.0001$) and time ($F(4,120)=126.827$, $p < 0.0001$) were detected, as well as an interaction between sex and time ($F(4,120)=16.370$, $p < 0.0001$). Baseline corticosterone was not different among neonatal stress groups, nor between males and females (Fig 4a). Immediately after restraint ($t=0$) both sexes displayed a significant elevation in plasma corticosterone ($p < 0.0001$), with females having significantly more corticosterone than males ($p < 0.0001$). Plasma corticosterone remained elevated above pre-restraint levels 30 min after release from restraint ($p < 0.0001$). Neonatal stress condition did not influence plasma corticosterone at any time after restraint. Handled females had greater overall plasma corticosterone compared to with-dam females as measured by area under the curve ($p < 0.05$, Fig. 4a inset), however there was no significant interaction between time and neonatal group.

Neonatal isolation did not affect plasma ACTH levels either at baseline or following 20 min restraint stress in either females (Fig. 5a) or males (Fig. 5b). There also were no significant differences between sexes. A significant effect of time was detected ($F(4,120)=68.671$, $p < 0.0001$) such that restraint significantly increased plasma ACTH relative to baseline levels (Fig. 5, $p < 0.0001$), and these levels remained elevated 30 min after release ($p < 0.001$ vs. baseline). Analysis of area under the curve did not indicate different integrated ACTH responses in neonatally isolated, handled or with-dam rats (Fig. 5 insets).

Discussion

Neonatal isolation enhanced anxiety in the open field following restraint stress

These data provide evidence that as few as 6 daily episodes of brief isolation from the dam, littermates, and all nest cues during the SHRP produce lasting changes in adult anxiety-related behavior. Following 1 hr restraint, neonatally isolated males and females selectively avoided the center of the open field compared to handled and with-dam littermates, and females exhibited less exploratory activity than with-dam controls. All groups traveled the same total distance, indicating that aversion to the center in neonatally isolated rats is not due to a generalized suppression of locomotor function. These findings extend previous work showing long-term effects of repeated episodes of protracted (i.e., 3-6 hr) separation of rat litters from maternal care (reviewed in Ladd et al., 2000; Holmes et al., 2005) and suggest that as little as 1 hr of daily isolation is sufficient to impact adult behavior.

The open field experiment suggests that adult rats subjected to brief neonatal isolation may not demonstrate altered behavioral responses to anxiety- or fear-provoking stimuli until they are stressed. This idea is supported by findings from a similar neonatal isolation paradigm that used a between-litter design to isolate pups 1 hr daily from P2-9. Those studies demonstrated that neonatal isolation did not alter basal locomotion, but (1) enhanced amphetamine-induced locomotor activation in juveniles (Kehoe et al 1996), (2) enhanced restraint-induced locomotor

suppression in adults (Kehoe et al 1998), and (3) attenuated restraint-induced locomotor activation in juveniles (McCormick et al 2002). Neonatal isolation also tended to increase behavioral sensitivity to foot shock after adult rats had been previously exposed to foot shock but did not alter the same behavioral responses when first presented during tone-shock conditioning (Kosten et al 2005). As in the current study, neonatal isolation did not alter time spent exploring the center of a novel open field (Kosten et al 2005). This pattern may be true for animals exposed to more extensive neonatal stress as well. Estanislau and Morato (2005) reported that as adults, maternally separated rats in which the entire litter was removed for 3 hr daily from P3-14 showed increased anxiety-related behavior on the EPM only after 1 hr restraint stress.

We cannot rule out the possibility that neonatal isolation influences habituation or sensitization to anxiety-provoking stimuli. That is, the neonatally isolated rats could be either habituating less or sensitizing more to anxiety-provoking stimuli upon repeated presentations, with prior restraint stress having no direct effect. Because we did not measure open field behavior in isolated rats exposed to the open field a second time without an intervening episode of restraint stress, we cannot distinguish among these possibilities. However, because neonatal isolation did not influence habituation to the center over the 10-15 min test period in either the first or second open field test, it is unlikely that center aversion in these rats is due to differential habituation.

Early life manipulation altered anxiety in the EPM

In the EPM, neonatally isolated rats spent less time in the open arms and made fewer open arm entries than handled rats but did not differ significantly from with-dam controls. Therefore, it is unclear whether these findings suggest increased anxiety in isolated rats or decreased anxiety in handled rats. Decreased anxiety in the EPM in neonatally handled rats would be consistent with a considerable body of literature demonstrating attenuated anxiety in these rats (Meaney et al., 1991; Anisman et al., 1998). To our knowledge, the current work is the only study to demonstrate an effect of neonatal handling on adult behavior in the EPM using a within-litter design.

Use of the within-litter design

The enduring effects of early handling are often attributed to altered maternal care when the dam is reunited with pups after a brief period of time with no pups present. To examine the effects of brief, repeated isolation unrelated to maternal care, we used a within-litter design in which dams were never left completely without pups. In this way all pups experience equivalent basal levels of environmental disruption, ensuring that the reported results are due specifically to daily isolation or handling, rather than daily nest disruption *per se*. Additionally, the within-litter design controls for individual differences in maternal behavior. There is ample evidence (Francis et al., 1996; Liu et al., 1997; Francis et al., 1999a; Francis et al., 1999b; Francis et al., 2002b; Meaney and Szyf, 2005) that natural variations in specific aspects of maternal care exert significant long-term effects on the stress and anxiety responses of offspring. We did not directly observe maternal behavior after returning pups to the nest, to avoid further nest disruption; however, by incorporating each experimental group into each litter, natural variations in maternal care should impact equally on all experimental groups. Thus, any enduring effect of handling or isolation in the current studies most likely represents a direct effect of the manipulation rather than an indirect effect on maternal behavior.

Neonatal isolation may not alter adult endocrine stress responses

It is interesting that neonatally isolated rats exhibited an apparent dissociation between their behavioral and endocrine stress responses. This contrasts with a previous study demonstrating enhanced corticosterone responses after 60 min restraint in juveniles exposed to neonatal

isolation stress (McCormick *et al.*, 2002). This difference may be explained by procedural differences between the prior study and the current work, such as more isolation episodes starting earlier in development (P2-9), a lack of bedding during isolation, the age at which HPA responses were examined, and the between-litter design used in the previous study. The current findings are, however, consistent with work in non-human primates ((Levine, 2005)) and suggest that neonatal isolation may selectively alter neural circuitry mediating behavioral stress responses in adulthood, such as the central nucleus of the amygdala (CeA). The CeA is the primary site of extrahypothalamic CRF production, a key mediator of behavioral stress responses (Heinrichs *et al.*, 1992; Swiergiel *et al.*, 1993; Lee and Davis, 1997; Petrovich and Swanson, 1997), and the site of the earliest identified neurobiological change after repeated neonatal handling (Fenoglio *et al.*, 2004). Plotsky *et al.* (2005) demonstrated that 3 hr of daily maternal separation from P2-14 significantly increased CRF protein expression in the adult CeA and altered CRF₁ receptor expression in the region, suggesting that maternal separation alters central CRF set points and allows enhanced hormonal and behavioral stress responses in adulthood.

Interpretation of the HPA response to restraint stress in the current experiments may be limited by the nature of the stressor. Francis *et al.* (2002a) demonstrated greater corticosterone elevations following 20 min restraint in adult offspring subjected to maternal separation (3 hr daily P1-14) than handled controls. However, many studies demonstrating altered stress responses following maternal separation utilize less severe stressors, such as air puff startle (Huot *et al.* 2001, Huot *et al.* 2001, Ladd *et al.* 2004) or foot shock (Ladd *et al.* 1996). Thus, the HPA response elicited in the current work may represent a maximal stress response that masks subtle between-group differences. In addition, plasma samples were taken after the second exposure to restraint stress, with the first exposure (prior to the second open field test) lasting 60 min. It possible that between-group differences existed on the first exposure, but that all rats may have habituated to the same level by the second presentation.

In conclusion, the current studies show that the comparatively mild stress of 6 daily, 1 hr episodes of isolation produces significant augmentation of anxiety-related behaviors in adulthood. This is consistent with work from other laboratories showing a spectrum of altered behavior in similarly treated rats. Because the behavioral changes were not accompanied by alterations in baseline or restraint stress-induced levels of ACTH or corticosterone, the findings suggest that neonatal isolation can modify adult behavior without significantly changing HPA responsiveness. Further studies are needed to determine whether HPA axis changes may be evident with novel stressors or stressors of different intensities.

Methods

General Methods

Animals—Fifty-five offspring of 8 Sprague-Dawley female rats (Taconic Farms, Germantown, NY) were used (mean 11.4 pups per litter; litters of less than 6 pups were excluded). Pregnant females were obtained between embryonic day (E) 10-14 and housed in reverse light-dark conditions (lights off 11:00-21:00). All dams were housed in individual plastic delivery cages and allowed ad libitum access to food and water. Dams were left undisturbed aside from regular cage maintenance until E21, when their cages were checked twice daily for the presence of pups. The day of birth was designated P0, during which all pups were counted, checked for evidence of feeding (i.e., visible milk band in abdomen), and any dead pups were removed. Litters were then left undisturbed aside from regular cage maintenance until the first day of manipulation. Individual pups within a litter were assigned to different conditions such that each experimental group included animals from multiple litters (within-litter design). The within-litter design was chosen to minimize the effect of differences in maternal care exhibited by individual dams. All rats were weaned in same-sex groups of 2-3

at P28-30 and left undisturbed until the onset of behavior testing on P66-68. All experimental manipulations (neonatal stress, adult behavior testing and blood sampling) occurred 0.5-4 hr after lights off. Experimental protocols were approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

Neonatal stress procedure

On P4 pups within a litter were randomly assigned to one of three stress groups, isolated, handled, and with-dam. The *isolated* pups were removed from their home cage, marked with indelible marker and isolated for 1 hr every day from P4-9. Individual isolated pups were placed alone in a clean plastic box containing an isothermal heating pad warmed to 35-37°C and clean bedding. Pups in the *handled* group were similarly removed and marked but were returned to their home cage rather than being isolated (total time away from the nest < 5 min). Because at least some of the effects of early manipulation are due to altered maternal care secondary to being without pups for 15 min or more, care was taken to minimize maternal stress, including minimal handling and leaving at least 3 pups with the dam at all times. These pups constituted the *with-dam* group. On P9 all pups were tattooed to identify their treatment group, and litters were then left undisturbed with the exception of routine cage maintenance until weaning.

Elevated plus maze (EPM)

Behavior testing began on P66-68, at which time adult rats were first tested for anxiety-related behavior on the EPM. Beginning 30 min after lights off each rat was placed in the center of the maze facing an open arm. Each arm measured 10 cm wide by 50 cm long. Walls of the closed arms measured 40 cm high, and the maze was elevated 72 cm from the floor. Their behavior was videotaped for 5 min under dim red illumination (light intensity = 30 lux in the center). After 5 min each rat was returned to its home cage, and the maze was cleaned with 70% ethanol. An observer blind to treatment groups scored videotape for time spent in open and closed arms and the number of open and closed arm entries.

Behavior in the open field

Between 3-11 d after EPM testing rats were placed in a 68.5 x 68.5 x 91 cm open field apparatus under dim red illumination (light intensity = 3 lux at the floor). Each rat was removed from its home cage and placed in one corner of the open field, and behavior was videotaped for 10 min. Behavior for two 5-min epochs was analyzed by an observer blind to neonatal condition and sex using a combination of computerized and manual scoring. Computerized scoring of distance traveled, velocity, and time in the center was accomplished using Ethovision software (version 2.3, Noldus, Amsterdam, The Netherlands). Grid crosses to the center, time spent grooming, and the number of rears were scored manually. The resultant data were divided into three categories of behavior: general motor activity (distance traveled, velocity), anxiety-related behavior (time spent in the center, grid crosses to the center, distance traveled in the center, and velocity in the center), and exploratory activity (number of rears).

Behavior in the open field after restraint stress

On the day after initial exposure to the open field rats were restrained in a flexible plastic restrainer (DecapiCone, Braintree Scientific, Braintree MA) for 1 hr prior to being placed in the open field. Behavior was recorded for 15 min and analyzed in three 5-min epochs as described above.

Jugular vein catheterization and plasma collection

After completion of all behavior testing rats underwent surgical implantation of plastic catheters in the right jugular vein. Three to 4 d after surgery serial blood samples (300 µl) were collected beginning 1 hr after lights off. Following a baseline (pre-restraint) sample each rat

was restrained for 20 min in the same manner as for the open field experiments. Upon release from restraint a second sample was taken (time = 0 min), and rats were returned to their home cage. Three additional recovery samples were collected at 30 min intervals for a total of 5 samples. Rats whose catheters were not patent at the time of the baseline blood collection were not used in these experiments. Samples were collected into a tuberculin syringe containing 10 μ l EDTA and transferred to a 0.5 ml polyethylene tube containing 10 μ l aprotinin. Each rat was given 100 μ l normal saline after sample collection to protect against fluid loss during the experiment. Samples were immediately centrifuged for 30 sec, and plasma aliquots were frozen on dry ice and stored at -80 °C until the time of assay. Plasma ACTH and corticosterone were determined in each sample, and the integrated hormone response (area under the curve) was calculated for each animal.

Corticosterone enzyme immunoassay

Plasma corticosterone was determined by enzyme immunoassay using CorrelateEIA Corticosterone kits (Assay Designs, Inc., Ann Arbor, MI) with modification. Plasma samples were thawed to room temperature and lightly vortexed. Ten μ l of plasma were pipetted in duplicate into clean, labeled microcentrifuge tubes and allowed to react for 5 minutes with dilute (1:100) steroid displacement buffer. This mixture was then diluted 1:40 and assayed according to kit instructions. Upon completion of the assay 96-well plates were read at 405 nm on a Spectra Max Plus microplate reader (Molecular Devices, Sunnyvale, CA) using SOFT max Pro 2.1.1 (Molecular Devices). Assay sensitivity was 27.0 pg/ml, and inter- and intra-assay variability were 17.0% and 6.2%, respectively.

ACTH radioimmunoassay

Plasma ACTH was determined in duplicate using 50 μ l of thawed plasma samples with an ¹²⁵I-ACTH radioimmunoassay kit (ICN Biomedical, Inc., Orangeburg, NY) with slight modification. Total kit volume was reduced by half to accommodate smaller sample sizes. Inter- and intra-assay variability were 14.0% and 11.0%, respectively.

Statistical analysis

Two-factor (sex $\times$ neonatal group) ANOVAs were used to analyze behavioral measurements over an entire test period and area under the curve, followed by Fisher's PLSD post-hoc tests. Three-factor (sex $\times$ neonatal group $\times$ time) repeated measures ANOVAs were used to compare differences across epochs of behavior and hormone as a function of time, followed by Bonferroni/Dunn post-hoc tests. A p value of less than 0.05 was considered statistically significant.

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Figure 1.

Behavior in the EPM. Behavior in the EPM was recorded for 5 min in adult rats that were isolated, handled, or with-dam on P4-9. Neonatal isolation significantly decreased the fraction of time spent in open arms (open/open+closed) (a), as well as open arm entries (b) compared to handled rats. Handled rats also make a higher proportion of open arm entries than with-dam rats (b). Data were collapsed across sex because there were no significant differences between males and females. * significantly different from handled controls ($p < 0.05$); ** significantly different from with-dam controls ($p < 0.05$). Values represent mean $\pm$ SEM. $n = 16-19$ per group.

Figure 2.

General motor activity in the open field following stressful challenge (1 hr restraint) in adult rats that were isolated, handled, or with-dam on P4-9. Adult rats were challenged with 1 hr restraint stress prior to being placed in the open field for 15 min. Neonatal stress condition had no influence on total distance traveled (a) or average velocity (b) in the open field after stressful challenge. —*— denotes a main effect of sex ($p < 0.0001$). Values represent mean $\pm$ SEM for the 15-min test period. $n = 8-10$ per group.

Figure 3.

Anxiety-related behavior in the open field following a stressful challenge. Activity in the center of an open field and rearing were recorded following 1 hr restraint in adult rats that were isolated, handled, or with-dam on P4-9. Neonatal isolation significantly decreased time in the center compared to handled and with-dam rats (a), grid crosses to the center compared to with-dam rats (b), and distance traveled in the center compared to with-dam rats (c). Isolated females exhibited less exploratory activity than with-dam females (d). * significantly different from handled controls ($p < 0.05$); ** significantly different from with-dam controls ($p < 0.05$). —*— denotes a significant effect of sex ($p < 0.05$). Values represent mean $\pm$ SEM for the 15-min test period. $n = 8-10$ per group.

Figure 4.

Neonatal isolation did not influence basal or stress-induced plasma corticosterone. Basal and stress-induced levels of plasma corticosterone were measured in adult rats that were isolated (black circles), handled (gray squares), or with-dam (open triangles) on P4-9. Restraint stress (20 min) increased corticosterone in females (a) and males (b) immediately after restraint (0 min), and levels remained high for 30 min after release from restraint (* $p < 0.0001$ vs. basal). Females had more corticosterone than males at 0 min ($p < 0.0001$). Females handled as neonates had higher integrated corticosterone secretion than with-dam females (a, inset, ** $p < 0.05$), an effect not detected in males (b, inset). Values represent mean $\pm$ SEM. $n = 5-7$ per group. AUC values expressed as ng/ml•min.

Figure 5.

Neonatal isolation did not influence baseline or stress-induced plasma ACTH. Basal and stress-induced levels of plasma ACTH were measured in adult rats that were isolated (black circles), handled (gray squares), or with-dam (open triangles) on P4-9. Restraint stress (20 min) increased ACTH in females (a) and males (b) immediately after restraint (0 min), and levels remained high for 30 min after release from restraint (* $p < 0.001$ vs. basal). Females and males displayed equivalent plasma ACTH at all time points, as well as equivalent integrated ACTH release across neonatal stress groups (insets). Values represent mean $\pm$ SEM. $n=5-7$ per group. AUC values expressed as pg/ml•min.

Table 1

Neonatal isolation does not affect activity in the novel open field.

		With-dam	Handled	Isolated
General Motor Activity				
Females	n	8	8	10
	Distance Traveled (cm)	5305.4 ± 356.6	5040.8 ± 418.9	4835 ± 187.4
	Velocity (cm/sec)	17.9 ± 0.5	17.2 ± 0.8	17.0 ± 0.4
Males	n	9	8	9
	Distance Traveled (cm)	4040.6 ± 319	4032.7 ± 286.7	4405.5 ± 322.3
	Velocity (cm/sec)	14.7 ± 0.7	15.5 ± 0.5	16.1 ± 0.6
Anxiety-Related Activity				
Females	n	8	8	10
	Time in Center (sec)	43.3 ± 6.9	46.3 ± 5.8	42.5 ± 7.8
	Crosses to Center	36.5 ± 4.6	39.6 ± 6.3	35.4 ± 4.7
	Distance in Center (cm)	675.5 ± 85.9	695.4 ± 88.7	667.1 ± 106.0
	Velocity in Center (cm/sec)	23.2 ± 1.2	23.0 ± 1.9	24.1 ± 1.4
Males	n	9	8	9
	Time in Center (sec)	40.6 ± 5.0	32.3 ± 7.0	27.4 ± 4.8
	Crosses to Center	33.1 ± 3.8	23.1 ± 4.7	24.3 ± 4.3
	Distance in Center (cm)	559.0 ± 89.4	410.3 ± 86.5	444.4 ± 82.1
	Velocity in Center (cm/sec)	21.1 ± 1.2	20.2 ± 1.8	19.7 ± 2.5
Exploratory Activity (rearing)				
		Females	47.3 ± 6.4	43.8 ± 2.8
		Males	29.6 ± 5.7	33.8 ± 3.6

General motor and anxiety-related activity during 10 min in an open field was recorded in adult rats that were isolated, handled, or with-dam on P4-9. In all cases values for females are greater than for their male counterparts ($p < 0.05$). Neonatal isolation had no effect on any behavior. Values represent mean ± SEM.