Research Report

Prenatal restraint stress and motherless rearing disrupts expression of plasticity markers and stress-induced corticosterone release in adult female Sprague–Dawley rats

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ABSTRACT

This study investigated the effects of prenatal stress and complete maternal deprivation, using the artificial rearing (AR) paradigm, on the expression of neural plasticity markers and hypothalamic–pituitary–adrenal (HPA) axis responsivity to stress. Rats were exposed to stress during gestation (day 10–21) and postnatally were either artificially reared (AR) or mother reared (MR). AR involves complete separation of the pup from both the dam and the litter throughout the pre-weaning period. In adulthood, we measured levels of corticosterone (CORT) in response to restraint stress. Also, we examined the expression of synaptophysin (SYN) and brain-derived neurotrophic factor (BDNF) in the medial prefrontal cortex (MPFC) and the nucleus accumbens (Nacc), areas of the brain that mediate behavioral activation and attention, among other behaviors. Earlier work on the same rats indicated that these behavioral endpoints, such as locomotor activity and sensorimotor gating, are affected by our prenatal and postnatal manipulations. Prenatal stress decreased CORT at 20 and 90 min post-stressor in MR, but not in AR, animals. Also, in comparison to MR groups, AR decreased SYN and BDNF expression in the MPFC and Nacc. Additional somatosensory ‘licking-like’ stroking stimulation partially reversed the effects of AR. Prenatal stress did not have a robust main effect but affected the impact of the postnatal rearing condition on SYN expression and stress-induced CORT. These results suggest that both prenatal and postnatal adversities have an influence on HPA axis responsivity and alter the expression of plasticity related neuronal proteins.

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Abbreviations: AR, artificial rearing; MR, mother reared; AR-MIN, artificially reared with minimal stimulation for AR-MIN; AR-MAX, artificially reared with additional somatosensory stimulation; CORT, corticosterone; PS, prenatal stress; NS, nonstressed; HPA, hypothalamic–pituitary–adrenal; SYN, synaptophysin; BDNF, brain-derived neurotrophic factor; MPFC, medial prefrontal cortex; Nacc, nucleus accumbens; CPu, caudate putamen; GD, gestational day; PND, postnatal day

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1. Introduction

Environmental factors play a pivotal role in shaping the central nervous system (CNS). Both prenatal and postnatal experiences shape the adult brain (Patin et al., 2002; Van Den Hove et al., 2006; Zhang et al., 2004). Prenatal experience is almost exclusively mediated by the mother’s behavioral and hormonal profile, and provides the background for subsequent postnatal environmental effects. The primary focus of this study was to examine the effects of prenatal stress and its interaction with postnatal maternal experiences on the expression of plasticity markers in adulthood. Since alterations in hypothalamic–pituitary–adrenal (HPA) axis function have been linked to disrupted expression of markers of plasticity (Bisagno et al., 2004; Jacobsen and Mork, 2006; Thome et al., 2001), we also examined the reactivity of the adult HPA axis. Previous behavioral findings from the same group of animals showed effects of postnatal maternal deprivation, and to a lesser degree prenatal stress, on measures of attention, activity, and emotion (Burton et al., 2006). Thus, a second focus of this study was to relate the behavioral changes observed in the same group of rats with changes in the expression of brain markers of plasticity and HPA function.

Prenatal stress produces a wide range of physiological and behavioral alterations in the adult offspring. One example is the effect of prenatal stress on the expression of neuronal markers of plasticity, such as synaptophysin (SYN) and brain-derived neurotrophic factor (BDNF). Prenatal stress alters synapse development as measured by decreased SYN expression, an integral protein of the synaptic vesicle that is involved in neurotransmitter release (Ishimaru et al., 2001). The expression of this protein is commonly used as an estimate of the number of functional synapses in a vesicle that is involved in neurotransmitter release (Ishimaru et al., 2001). BDNF is another marker of plasticity, which is also decreased by prenatal stress (Van Den Hove et al., 2006). BDNF is a trophic factor, similar to nerve growth factor (NGF), known to be involved in cell survival as well as synaptic plasticity (Lewin and Barde, 1996). These disruptions of markers of neuronal and synaptic plasticity are likely involved in the changes in behavior observed in prenatally stressed animals.

One potential contributor to the effects of prenatal stress on SYN and BDNF expression is dysregulation of the HPA axis (Bisagno et al., 2004; Jacobsen and Mork, 2006). The effects of prenatal stress on HPA reactivity, specifically corticosterone (CORT) release, are inconsistent for female rats. Some studies demonstrated that prenatal stress increases stress-induced CORT (McCormick et al., 1995; Szuran et al., 2000), while others have found no effect (Bhatnagar et al., 2005; Virgolini et al., 2006). Thus, the effects of prenatal stress on HPA function in females are still unclear, particularly in the context of its mediating effect on the brain.

Postnatal experience also plays an important role in the development of the CNS. The artificial rearing (AR) paradigm (Hall, 1975) has been used to assess the effects of the neonatal environment on the brain and behavior. AR involves complete separation of the pup from both the dam and the litter throughout the pre-weaning period. It produces a wide range of behavioral alterations (Chatterjee et al., 2007; Gonzalez et al., 2001; Lovic and Fleming, 2004). These include disrupted maternal and social behavior, attention, and emotionality (Burton et al., 2006; Gonzalez et al., 2001; Lovic and Fleming, 2004; Novakov and Fleming, 2005). However, providing AR rats with additional somatosensory, maternal licking-like stimulation either completely or partially reverses these effects in most cases (Burton et al., 2006; Gonzalez et al., 2001; Lovic and Fleming, 2004; Novakov and Fleming, 2005). Although the behavior of AR rats has been thoroughly investigated (Burton et al., 2006; Gonzalez et al., 2001; Lovic and Fleming, 2004; Novakov and Fleming, 2005), less is known about the impact of AR on the brain as well as the HPA axis.

Recently, our group demonstrated that AR alters brain plasticity. We found that AR decreased the expression of various markers of plasticity such as SYN, and BDNF in various brain areas in male rats (Chatterjee et al., 2007). Chatterjee et al. (2007) suggest that these characteristics of AR rats may be involved in the observed disruptions in behavior, although these investigators did not measure behavior in their animals. Furthermore, this decrease in neural plasticity is likely involved in the disruptions in behavior that have been reported previously (Burton et al., 2006; Gonzalez et al., 2001; Lovic and Fleming, 2004; Novakov and Fleming, 2005).

Although many studies have investigated the effects of either pre- or postnatal adversity, less is known about how these factors interact. Studies in this area typically attempt to reverse the effects of prenatal insult. For example, both prenatal handling and enriched environments have been shown to reverse the effects of prenatal restraint stress on BDNF and SYN expression (Bredy et al., 2004; Koo et al., 2003; Lemaire et al., 2006). In terms of behavior, recently, we reported that prenatal stress and AR produce alterations in attention and locomotor activity (Burton et al., 2006). The effects of these manipulations were not additive. However, there was a clear interaction between prenatal and postnatal adversity on locomotor activity. Providing AR rats with additional licking-like stimulation (AR-MAX) ‘normalized’ (decreased) locomotion in non-prenatally stressed rats, such that they resembled their mother reared counterparts. In contrast, prenatally stressed AR-MAX rats had the highest level of locomotor activity. Thus it appears that additional somatosensory stimulation can have opposing effects depending on the rat’s prenatal experience and the dependent measure. These results indicate that the effects of postnatal experience can depend on the prior background of the animal being stimulated.

The current study expanded on our behavioral findings (Burton et al., 2006) and histological findings (Chatterjee et al., 2007) by assessing the effects of prenatal stress and its interaction with AR on the expression of plasticity markers, such as SYN, and BDNF and stress-induced CORT release in female rats. Neuroplasticity and the HPA axis have been linked to disruptions in attention and locomotor activity (Ingram et al., 2005; Marín et al., 2007; Varty et al., 1999) as observed previously in this same group of rats (Burton et al., 2006). The presence of the plasticity markers was examined in the medial prefrontal cortex (MPFC), nucleus accumbens (Nacc), and caudate...
2. Results

No significant differences between MR-SHAM and MR-CON groups observed for any measure, thus these groups were combined and are referred to as MR.

2.1. Basal and stress-induced serum corticosterone

We observed a significant effect of sample time, $F(1,37)=134.477$, $p=0.0001$. CORT was increased at 20 min for all rats and then increased or decreased at 90 min. There were no overall main effects of prenatal stress or of rearing condition across time points. However, there was a significant prenatal stress $\times$ sample time interaction, $F(1,37)=7.286$, $p=0.010$. At baseline, there was a non-significant trend towards prenatally stressed rats having higher serum CORT levels, $F(1,37)=2.996$, $p=0.092$. At 20 min, however, there was a significant main effect of prenatal stress, $F(1,37)=5.208$, $p=0.028$, with prenatally stressed rats having lower serum CORT than nonstressed rats. Again, at 90 min, there was a non-significant trend towards prenatally stressed rats having lower serum CORT levels $F(1,37)=3.545$, $p=0.068$. In addition, we observed a significant time $\times$ stress $\times$ condition interaction, $F(2,37)=3.999$, $p=0.027$. This effect was driven by a significant interaction between prenatal stress and rearing at 90 min, $F(2,37)=4.667$, $p=0.016$. A one-way ANOVA with a Tukey’s post hoc test revealed that at this time point, prenatally stressed MR rats had significantly lower serum CORT than nonstressed MR rats ($p=0.003$) (see Fig. 1).

2.2. Synaptophysin

2.2.1. MPFC

There was a significant main effect of prenatal stress, $F(1,37)=4.395$, $p=0.043$. Prenatally stressed rats had significantly less SYN expression than nonstressed rats. Further, there was a significant main effect of rearing, $F(2,37)=196.495$, $p=0.0001$. All groups were significantly different from each other ($ps<0.003$) with AR-MIN rats having the least SYN expression and MR having the greatest. Moreover, we observed a significant interaction between prenatal condition and postnatal rearing condition, $F(2,37)=17.950$, $p=0.0001$. Prenatal stress decreased SYN expression in AR rats, but increased SYN expression in MR rats (see Fig. 2a).

2.2.2. Nacc

There was a marginal main effect of prenatal stress, $F(1,37)=3.755$, $p=0.060$. Prenatally stressed rats had less SYN expression than nonstressed rats. Further, there was a significant main effect of rearing, $F(2,37)=73.059$, $p=0.0001$. All groups were significantly different from each other ($ps<0.004$) with AR-MIN rats having the least SYN expression and MR having the greatest. Moreover, we observed a significant interaction between prenatal condition and postnatal rearing condition, $F(2,37)=19.667$, $p=0.0001$. Prenatal stress decreased SYN expression in AR rats, but increased SYN expression in MR rats (see Fig. 2b).

2.2.3. Cpu

There were no main effects or interactions of prenatal stress or rearing (see Fig. 2c).

2.3. BDNF

2.3.1. MPFC

There was a significant main effect of prenatal stress, $F(1,37)=9.833$, $p=0.003$. Prenatally stressed rats had significantly less BDNF expression than nonstressed rats. Furthermore, there was a significant main effect of rearing, $F(2,37)=84.283$, $p=0.0001$. All groups were significantly different from each
other (ps<0.0001) with AR-MIN rats having the least BDNF expression and MR having the greatest. Moreover, we observed a significant interaction between prenatal condition and postnatal rearing conditions, F(2,37)=6.706, p=0.003. Prenatal stress decreased BDNF expression in AR-MAX rats (a, p<0.05), but increased BDNF expression in MR rats (b, p<0.05). Panel b shows that there was a significant main effect of rearing in the Nacc and all rearing groups were significantly different from each other. AR-MIN rats had the least BDNF expression and MR had the greatest. Furthermore, prenatal stress does not have a robust main effect on BDNF expression; however, there was a significant interaction between prenatal and postnatal rearing conditions. Prenatal stress decreased BDNF expression in AR-MIN rats (a, p<0.05), but increased BDNF expression in MR rats (b, p<0.05). In the CPu (Panel c), there were no main effects or interactions of prenatal stress or rearing. Western blot pictures for SYN are shown in panel d. Prenatal stress=PS, nonstressed=NS, AR-MIN=artificially reared with minimal stroking stimulation, AR-MAX=artificially reared with maximal stroking stimulation, MR=mother reared. PS AR-MIN, n=6, NS AR-MIN, n=4, PS AR-MAX, n=6, NS AR-MIN, n=4, PS MR, n=8, NS MR, n=13.

2.4. Behaviors

2.4.1. Prepulse inhibition and activity
Although the present paper only reports on the physiological markers, Figs. 4 and 5 (from Burton et al., 2006) are included to show how this same group of rats performed earlier on measures of behavioral attention and activation.

2.5. Correlations

2.5.1. Markers of plasticity, CORT, and behavior
Bivariate correlations demonstrated significant correlations between both markers of plasticity, and CORT with our behavioral measures (Burton et al., 2006). Rats with lower SYN...
and BDNF expression in both the MPFC and Nacc also exhibited higher locomotor activity and lower PPI (the significant \( r \) values ranged from \( r = 0.28 \) to \( 0.88 \), \( p < 0.05 \) to 0.001). Also, rats with lower CORT at 20 min post-stressor had increased BDNF expression in the MPFC and Nacc (\( r = 0.349, p < 0.022 \) and \( r = 0.301, p < 0.05 \), respectively). However, when partial correlations were conducted covarying prenatal and postnatal conditions, these correlations were no longer significant.

2.5.2. Markers of plasticity and CORT

There were significant positive correlations between CORT and BDNF; animals with higher BDNF in MPFC or in Nacc had higher CORT levels at 90 min post-stressor (\( r = 0.34, p < 0.022 \), and \( r = 0.30, p < 0.05 \), respectively). As before, this effect was eliminated after group covariation. However, the correlation between CORT and SYN was retained after covarying group. SYN expression in the Nacc was significantly negatively correlated with serum CORT at 20 min and 90 min post-stressor (\( r = -0.332, p = 0.034 \) and \( r = -0.543, p = 0.0001 \), respectively). Furthermore, SYN expression in the MPFC was negatively correlated with CORT (\( r = -0.396, p = 0.010 \)). Therefore, rats with greater serum CORT in response to a stressor have decreased SYN expression in the MPFC and the Nacc.

3. Discussion

This study examined the effects of prenatal stress and AR on the expression of brain plasticity markers and stress-induced CORT. With respect to the brain markers, we observed a significant effect of AR on SYN and BDNF expression in the MPFC and Nacc. Consistent with previous work (Chatterjee et al., 2007), AR with minimal somatosensory stimulation (AR-MIN) decreased SYN and BDNF expression. Additional somatosensory stimulation (AR-MAX) partially reversed the effects of AR. Similarly to the behavioral data from the same group of rats (Burton et al., 2006), prenatal stress had a
differential effect on SYN depending on the rat’s postnatal experiences. Prenatal stress only decreased SYN expression in the MPFC and Nacc in AR rats, but increased SYN expression in MR rats. Also, prenatal stress significantly decreased stress-induced CORT levels. Furthermore, at 90 min post-stressor, prenatally stress MR rats had significantly lower levels of CORT than nonstressed MR rats. Finally, rats with lower levels of SYN expression also had increased stress-induced CORT release. These findings suggest that early adversity disrupts the expression of SYN and BDNF perhaps through altered HPA function.

3.1. Prenatal and postnatal manipulations

3.1.1. Molecular markers
Consistent with our behavioral data, we did not observe a robust main effect of prenatal stress on markers of brain plasticity in the MPFC and Nacc as was shown in a previous study (Fumagalli et al., 2004). This lack of effect is likely related to the method of prenatal stress (discussed below). With respect to AR, our findings are consistent with a previous study from our group (Chatterjee et al., 2007). AR decreased the expression of SYN and BDNF as compared with MR rats. Further, additional somatosensory stimulation (AR-MAX) partially reversed the effect of AR. Although our stroking stimulation does not replace maternal body licking, it simulates the somatosensory stimulation that pups would receive in the nest. Numerous studies have demonstrated that maternal licking impacts the development of both brain and behavior in the offspring (see Caldji et al., 2000; Liu et al., 2000 for example). In line with this evidence, neonatal somatosensory (stroking) stimulation has been demonstrated to facilitate growth in preterm infants (Field et al., 1986). Recently, Yokoyama and colleagues (2006) demonstrated that neonatal somatosensory/stroking stimulation increased synaptophysin in the rat somatosensory cortex, suggesting that neonatal somatosensory stimulation, which mimics maternal body licking, affects the physical and CNS development of the organism.

Another factor, which may play a role in the effect of AR on the expression of markers of brain plasticity, is the lack of social interaction with littermates and the dam. We have addressed this issue in a previous experiment (Melo et al., 2006). In this study, MR rats were compared with AR that received minimal stimulation (AR-MIN) and AR rats that had a same-sex conspecific in their cups and received tactile stimulation (like AR-MAX with a social partner). We found that the addition of a social partner and additional somatosensory stimulation partially reversed the effects of AR on maternal behavior, social behavior, and social learning. Unfortunately, we cannot dissociate the effects of a social partner from the additional somatosensory stimulation. However, these results are inline with previous studies that only included the somatosensory stimulation (AR-MAX; Gonzalez et al., 2001; Levy et al., 2003), which suggests that social interaction plays a relatively small role in the reversal of the effects of AR. These findings suggest that the effects of AR are not just the result of lack of social interaction during the pre-weaning period.

Fig. 4 – Shows the effects of the prenatal stress and artificial rearing from the same group of rats as published in Burton et al. (2006), a subset of which was included in the present paper. Maximal stimulation partially reversed the reduction in prepulse inhibition (PPI) produced by artificial rearing. PS=prenatally stressed; NS=prenatally nonstressed; AR-MIN=artificially reared with minimal stimulation; AR-MAX=artificially reared with maximal stimulation; MR=mother reared. PS AR-MIN, n=6, NS AR-MIN, n=6, PS AR-MAX, n=6, NS AR-MIN, n=4, PS MR, n=8, NS MR, n=13.

Fig. 5 – Shows the effects of the prenatal stress and artificial rearing from the same group of rats as published in Burton et al. (2006), a subset of which were included in the present paper. Prenatal stress and artificially rearing significantly increased locomotor activity. Providing AR rats with additional licking-like stimulation (AR-MAX) ‘normalized’ (decreased) locomotion in non-prenatally stressed rats, such that they resembled their mother reared counterparts. In contrast, prenatally stressed AR-MAX rats had the highest level of locomotor activity (a, p<0.05). Prenatal stress=PS, nonstressed=NS, AR-MIN=artificially reared with minimal stroking stimulation, AR-MAX=artificially reared with maximal stroking stimulation, MR=mother reared. PS AR-MIN, n=6, NS AR-MIN, n=6, PS AR-MAX, n=6, NS AR-MIN, n=4, PS MR, n=8, NS MR, n=13.
3.1.2. Corticosterone response to stress
In this study, prenatal stress reduced serum CORT in response to a stressor. This is a novel finding; however, the effects of prenatal stress on stress-induced CORT release have not been consistent (Bhatnagar et al., 2005; McCormick et al., 1995; Szuran et al., 2000; Virgolini et al., 2006). One possible explanation for these inconsistent results is rat strain differences between studies. Studies that found that prenatal stress increased stress-induced CORT in females used Long-Evans and Wistar rats. In contrast, in line with our findings and methodology, Bhatnager et al. (2005) also used Sprague-Dawley rats and found no effect of prenatal stress on stress-induced CORT. Thus the effect of prenatal stress on CORT is likely strain dependent. Another possible explanation for this finding is that previous studies used the standard restraint stress paradigm (see below). However, a recent study using a psychosocial stressor, which was considered a mild stressor, found that prenatal stress attenuated ACTH-induced CORT in males as compared with control rats (Gotz and Stefanski, 2007). In our study, the parameters of our restraint stress paradigm may have produced only mild stress in the dam and, in turn, her offspring.

The restraint stress paradigm used in this study was originally selected because it was expected to produce the most extreme effects (Fujikawa et al., 1999; Hashimoto et al., 2001). The most common paradigm of restraint stress is 3 45-min sessions daily from GD 14–21, which has been shown to increase levels of corticosterone and ACTH in the dam and her fetuses (Williams et al., 1999). Unfortunately, the effects of our restraint stress protocol (a single 4 h session occurring daily from GD 10 to 21) on the dam are presently unknown. Our assumption that this procedure would produce still greater stress effects than are found with shorter restraint periods may be unfounded. In fact, it may be the case that exposure to this repeated stressor produces adaptation and decreased HPA response to the stressor rather than an increase (see Bhatnagar and Dallman, 1998; Dhabhar et al., 1997). Furthermore, because our stressor was applied from GD 10 to 21, habituation may have occurred during the period when the offspring are most susceptible, that is, GD 14–21 (Koenig et al., 2002). In future studies, we plan to use variable stressors and stressors starting on GD 14, which may produce stronger prenatal stress effects (Koenig et al., 2005; Lehmann et al., 2000; Lemaire et al., 2000; Vallée et al., 1996). Therefore, this finding in conjunction with our results suggests that exposure to a mild stressor during gestation results in attenuating stress-induced CORT release.

Inconsistent with our hypothesis, there was no effect of AR on CORT. However, this finding is consistent with some maternal separation/deprivation studies (Bhatnagar et al., 2005; Virgolini et al., 2006). One possible explanation for our unexpected results is that we did not control for day of the estrus cycle. It can be assumed that because the rats from each group were evenly distributed across testing days that the day of the estrus cycle would be evenly distributed as well. However, the effects of AR on CORT may be subtle and the variability introduced by not controlling for the estrus cycle may have masked or eliminated the effects. Furthermore, not removing the rats from the room where the stress occurred may explain why we did not observe a reduction of CORT at 90 min in our control rats.

3.2. Interaction of prenatal and postnatal conditions
Our results indicate that prenatal experiences alter the impact of postnatal rearing condition on adult neuronal protein expression and stress-induced CORT release. These findings are consistent with the results of locomotor activity from the same group of rats (Burton et al., 2006). In addition, the nature of this interaction varies as a function of the type of neuronal protein. In terms of SYN expression, prenatally stressed MR rats had the highest levels of SYN in both the MPFC and Nacc, which suggests an increased density of functioning synapses. Thus it appears that prenatal stress increases SYN in MR rats, instead of decreasing SYN as was observed in AR rats. A similar interaction was observed in the stress-induced CORT release. The prenatal condition of MR rats differentially affected the return corticosterone levels to baseline. Prenatally stressed MR rats had significantly lower serum CORT 90 min-post-stressor as compared with nonstressed MR rats. These results suggest that prenatal stress improves negative feedback in the HPA axis in control rats, which is incongruent with previous findings (Vallée et al., 1999, 1996). As discussed previously, these effects may be the result of mild prenatal stress enhancing physiological function in conventionally reared rats.

One hypothesis for the cause of this dichotomous effect of ‘mild’ prenatal condition on the postnatal enhancement of HPA function and expression of SYN is variations in maternal behavior. Prenatally stressed rats may elicit more maternal behavior from their dams, which in turn may lead to alterations in HPA function and neuronal protein expression. This hypothesis is supported by previous studies. Muir et al. (1985) demonstrated that dams stressed during pregnancy spend more time in the nest and more time nursing. Furthermore, offspring from dams that spend more time licking, grooming, and nursing (High-LG mothers) exhibit a reduced CORT response in response to a stressor as well as return to baseline more quickly (Liu et al., 1997). Also, pups from High-LG mothers have increased SYN expression (Liu et al., 2000). Unfortunately, we did not observe the maternal behavior of our dams. However, the aforementioned studies suggest that the attenuated stress-induced CORT release and increased SYN expression observed in prenatally stressed MR rats may be due in part to increased maternal behavior.

The nature of the interaction between prenatal stress and AR was slightly different with respect to BDNF. Prenatal stress decreased BDNF expression in AR rats but did not have an effect on MR rats, as was observed with SYN expression. Liu and colleagues (2000) demonstrated that pups of High-LG dams have both increased SYN and BDNF expression. However, expression of BDNF was only measured in the hippocampus in the later study, thus this effect may not exist in the corticostriatral pathway.

3.3. Relationship between stress-induced corticosterone and neuronal protein expression
SYN expression was significantly correlated with stress-induced CORT release. Rats with low levels of SYN expression in both the MPFC and Nacc had a higher CORT response to a stressor and did not return to baseline as quickly. These
findings suggest that having fewer synapses are related to a dysfunctional HPA responsivity to stressor. Furthermore, these findings suggest that the effect of AR on SYN expression may be in part mediated by CORT.

Significant correlations were observed between the expression of BDNF and behavior as well as CORT. However, when partial correlations were conducted covarying prenatal and postnatal conditions, these correlations disappeared. One interpretation is that our measures of plasticity and CORT are not involved in sensorimotor gating, emotionality or locomotor activity. However, because both serum CORT collection and the time of sacrifice occurred considerably later than the behavioral measures, the lack of correlation may in fact be the result of temporal disparity between measures.

In summary, this study demonstrated that prenatal stress and AR, both separately and together impact both neural protein expression and HPA response to stress. Although these measures were not correlated with our behavioral measures when group status was covaried (Burton et al., 2006), these studies demonstrated that prenatal experiences can have an important impact on the potential effects of postnatal manipulations, depending on the outcome measure. Furthermore, they show that increased somatosensory stimulation through additional stroking stimulation can reverse in many cases the effects of complete maternal deprivation during the pre-weaning period. This finding is important because it demonstrates the plasticity of the CNS and the potential to at least partially reverse the effects of early adversity.

4. **Experimental procedures**

4.1. **Subjects**

A total of 43 female Sprague–Dawley rats were used in this study. The rats were born and raised at the University of Toronto at Mississauga from stock originally obtained from Charles River Farms (St. Constant, Quebec, Canada). The colony was maintained on a 12:12 h light:dark cycle with lights at 0800 h in a room maintained at approximately 22 °C, humidity 50–60%. Beginning on PND day 21, rats were housed two per cage (clear Plexiglas, 20 cm×43 cm×22 cm), with food (Purina Rat Chow) and water available ad libitum. All the procedures described in this report conformed to the guidelines set by the Canadian Council on Animal Care and in accordance with Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. The University of Toronto at Mississauga Local Animal Care Committee approved the procedures.

4.2. **Apparatus and procedure**

4.2.1. **Mating**

Vaginal smears were taken from 19 virgin females over several days at approximately 1400 h. Once the females were in proestrus, they were placed with a sexually experienced male for 24 h. Presence of spermatozoa in a vaginal smear after mating was considered gestational day (GD) 0.

4.2.2. **Prenatal stress**

Dams were left undisturbed until GD 10 at which point they were randomly assigned to PS or NS groups. From GD 10 to 21, PS dams were weighed and then placed in a Plexiglas restrainer (8 cm diameter×20 cm length) for 4 h per day at random times between 900 h and 1800 h. Restrainers were designed to limit, but not prohibit, movement of the pregnant female or to constrict her abdomen. The length of the chamber was flexible (15 to 18 cm) to accommodate the range of dam sizes. After each session, the restrainers were cleaned thoroughly with 30% alcohol. This type of stressor has been used previously (Fujikura et al., 1999; Hashimoto et al., 2001). Rats were monitored to ensure appropriate weight gain and any signs of poor health. NS dams were undisturbed.

4.2.3. **General procedures**

Dams were allowed to give birth undisturbed. On the day of parturition (PND 0), litters were culled to 14 rats (7 males and 7 females). On PND 4, 3 males and 3 female pups were removed from the nest and implanted with a cheek cannula (see Surgery and artificial rearing below). The two pups that would become MR-CON rats remained with the dam and the rest of the litter. One male and one female each received a sham surgery and was returned to the nest (MR-SHAM), and the remaining pups were artificially reared (AR) and randomly assigned to two groups (see Treatments and Groups below). For this study, only a subset of the female offspring was used. This resulted in the following group compositions: PS AR-MIN, n=6, PS AR-MAX, n=6, PS MR-SHAM, n=3, PS MR-CON, n=5, NS AR-MIN, n=6, NS AR-MAX, n=4, NS MR-SHAM, n=6, NS MR-CON, n=7. These rats were derived from 16 litters with only one rat from each litter per group.

4.2.4. **Surgery and artificial rearing**

Prior to surgery, pups were weighed and a topical anaesthetic (Eutectic Mixture of Local Anesthetics [EMLA], containing 2.5% lidocaine and 2.5% prilocaine) was applied to their right cheek. A leader wire (stainless steel 0.25 mm in diameter), sheathed in lubricated (mineral oil), silastic tubing and polyethylene (PE) 10 tubing was used to pierce the cheek. Once the flared end of the tubing contacted the inside of the cheek, the leader wire and silastic tubing were removed and Polysporin antibiotic ointment was applied topically to the site of penetration. Another leader wire was then used to insert a t-washer, which was secured in place with Super-glue. MR-SHAM pups had their cheeks pierced but the PE 10 tubing was removed. Polysporin was applied to the site of penetration and non-toxic permanent black marker was applied to the pups’ ears prior to placing them back with the litter for later identification.

After surgery, AR pups were placed individually in plastic cups (11 cm diameter×15 cm deep) containing corn cob bedding (Bed O’Cobs), each of which was placed inside another weighted cup. The cups floated in temperature controlled (34–37 °C) water directly below time-controlled infusion pumps (Harvard Apparatus Syringe, PHD 2000) to which they were connected with the cheek cannula tubing. The pumps delivered milk (Messer diet) for 10 min every hour, 24 h a day. The amount infused was calculated based on mean pup body weight. Beginning on PND 4, pups
received a volume of milk equal to 33% of the mean body weight, and this amount increased by 1% daily. Each morning, the pups were disconnected from the pumps, their weight was recorded, and all tubing was flushed with double distilled water. New syringes with fresh formula were prepared, and the new infusion rates were programmed based on the new weights.

4.2.5. Treatment and groups
AR rats were randomly assigned to either AR-MIN or AR-MAX groups. Each day, AR-MIN pups were stimulated twice for 30 s each (morning and night) with a wet camelhair paintbrush in the anogenital region to stimulate urination and defecation. AR-MAX pups also received the same anogenital stimulation twice daily, as well as 2 min of dorsal stimulation with a dry camelhair paintbrush 8 times a day. Stimulations for both groups occurred daily from PND 4 to 16. On PND 17–18, pups were removed from the pumps and given milk formula, rat chow, and a mixture of the two.

On PND 21, all rats were weighed and paired with a social partner of the same sex from another litter that was not tested. Rats were left undisturbed until PND 60 when they were tested on prepulse inhibition (PPI), and locomotor activity. All behavioral testing occurred during the light cycle. Methods for testing the PPI and locomotor activity are described in detail elsewhere (Burton et al., 2006).

4.2.6. Blood collection and radioimmunoassays
To collect baseline measures of CORT, rats were restrained and blood was collected from a nick in the tail into non-Hepranized capillary tubes (4 tubes; approximately 200 μl from each rat) and immediately placed on ice. After the blood was collected, the rat was placed into a Plexiglas restrainer (described above in Prenatal Stress section). Twenty minutes later, a second sample of blood was collected as stated above while the rat was in the restrainer. Immediately after blood collection, the rat was placed into its homecage without its anogenital stimulation twice daily, as well as 2 min of dorsal stimulation with a dry camelhair paintbrush 8 times a day. Stimulations for both groups occurred daily from PND 4 to 16. On PND 17–18, pups were removed from the pumps and given milk formula, rat chow, and a mixture of the two.

Blood was allowed to rest on ice for at least 30 min before being placed in the centrifuge. Blood was spun in a centrifuge (Eppendorf 5804R) at 4 °C, 4000 rpm for 20 min. Immediately after, the serum was extracted using a 3 ml transfer pipette and stored in 0.5 ml tubes at −80 °C. Finally, bloods were tested for levels of CORT using radioimmunoassays (RIA). Corticosterone was determined by a solid phase (I-125) radioimmunoassay (Coat-a-Count, Diagnostic Products Corporation; inter-assay variability=8.5%; intra-assay variability=6.83%).

4.2.7. Tissue preparation
All histological procedures were based on Chatterjee et al. (2007). Between 9 and 11 months of age, the rats were decapitated. The brains were immediately placed into isopentane for 30 s, then frozen with dry ice, and stored at −80 °C. The brains were fixed in 4% paraformaldehyde overnight, and 24 later, were dehydrated with successive solutions of 4% paraformaldehyde with 10, 20, and finally 30% sucrose in 1-h increments. Brains were stored in this final solution at 4 °C until sectioning. At the time of sectioning, brains were brought to −20 °C and successive 22 μm sections were taken with a cryostat (Microm HM 520, Richard-Allan Scientific, Kalamazoo, MI). The brain was sectioned throughout all the areas of interest and consecutive sections were mounted onto separate slides, such that both antibodies could stain each brain area and a number of sections across the anterior–posterior plain were collected from each brain site. Slides containing 5 to 6 slices each were immediately stored at −80 °C.

4.2.8. Western blots
Western blot methods and analysis were carried out as described by Chatterjee et al. (2007) and Miyake and colleagues (2002). Bilateral samples for the MPFC, the Nacc (shell and core combined), and CPu were collected from 322 μm sections selected based on landmarks from Paxinos and Watson (1986). The CPu was chosen as a control site based on a previous study from our group (Chatterjee et al., 2007). Tissue samples were pooled in 15 μl of 10 mM phosphate buffer. Samples were sonicated for 5 s at setting 2. Proteins were assayed using Pierce’s BCA protein assay reagent (PIERCE, Rockford, IL, USA). SDS-sample buffer (5 μl, 4 × concentration) was added to each sample. 10 μg of protein from each sample was loaded in each lane and separated by SDS-polyacrylamide gel electrophoresis in BioRad mini gels (BioRad, Hercules, CA). Separated proteins were transferred to PVDF membranes using a BioRad mini transblot apparatus. Membranes were incubated with monoclonal anti-synaptophysin antibody (detection of synaptophysin; dilution: 1:500; Sigma, St. Louis, MO), followed by HRP coupled anti-mouse IgG (Sigma Chemicals, St. Louis, MO; dilution 1:600). For BDNF, the same procedure was applied but the primary antibody was anti-BDNF polyclonal (rabbit polyclonal, dilution: 1:500; Chemicon, Temecula, CA) and the secondary antibody was HRP coupled anti-rabbit IgG (Sigma, St. Louis, MO, dilution: 1:600). The immunoreactivity was visualized by ECL technique according to the manufacturer’s instructions (GE Healthcare Bio-Sciences Corp., Cardiff, Wales) using the phospho-imager. Optical density of the bands was quantified with BioRad software (PD QUEST).

4.3. Statistical analyses
CORT data were analyzed using a 3 (time: baseline, 20 min post-stressor, 90 min post-stressor) × 2 (stress: prenatally stressed vs. nonstressed) × 3 (rearing: AR-MIN vs. AR-MAX vs. MR) repeated measures analysis of variance (ANOVA). Furthermore, univariate ANOVAs were conducted for each time point to establish the nature of the interactions. For both the SYN and BDNF data, the average optical density for each brain site was examined in a 2 (stress: prenatally stressed vs. prenatally nonstressed) × 3 (rearing: AR-MIN vs. AR-MAX vs. MR) univariate ANOVA. The Bonferroni test was used for post hoc analyses. Finally, correlations were conducted for molecular markers, CORT, and behavior. The level of statistical significance was p < 0.05.
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References


McCormick, C.M., Smythe, J.W., Sharma, S., Meaney, M.J., 1995. Sex-specific effects of prenatal stress on...


Rees, S.L., Steiner, M., Fleming, A.S., 2006. Early deprivation, but not maternal separation, attenuates rise in corticosterone levels after exposure to a novel environment in both juvenile and adult female rats. Behav. Brain Res. 175, 383–391.


