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Long-term behavioural and molecular alterations associated with maternal separation in rats

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Abstract

In this study we addressed whether certain behavioural measures, endocrine levels and specific stress-related proteins exhibit long-term alterations in adult rats following repeated postnatal maternal separation. Rats were subjected to daily maternal separation for 15 min (HMS15) or 180 min (HMS180) from postnatal day 2–14. Adult HMS180 animals were hypoactive and had increased levels of stereotypy compared to HMS15 and normal animal facility-reared (AFR) animals. HMS180 animals also had augmented plasma adrenocorticotropin (ACTH) and corticosterone (CORT) concentrations following an acute stressor, compared to the other two groups. We assessed persistent changes in proteins regulated by stress in hippocampus, cortex, ventral tegmental area, nucleus accumbens, striatum and amygdala. Western blotting analysis revealed a decrease in the levels of mature brain-derived neurotrophic factor (BDNF) in hippocampus and striatum, but an increase in the ventral tegmental area in the HMS180 rats. Levels of pro-BDNF were significantly increased in the ventral tegmental area of HMS180 animals but were unchanged in other brain regions compared to the other two groups. Levels of the transcription factors cAMP response element binding protein (CREB) and ΔFosB were unchanged in all of the brain regions studied in the maternally separated rats. These data show that maternal separation induces long-term changes in BDNF expression, and more specifically the processing of BDNF, in the hippocampus, striatum and ventral tegmental area. Recognition of these adaptations begins to define the brain regions, and neural circuitry, associated with persistent alterations induced by early life stressors and the development of mood disorders.

Introduction

Maternal separation has been proposed as an animal model of early life stress and the subsequent development of adult depression-related behaviour (Hall, 1998; Ladd *et al*., 2000; Arborelius *et al*., 2004). One of the most widely used paradigms for maternal separation involves subjecting neonatal rats from postnatal day (PND) 2 to 14 to daily maternal separation for 15 (HMS15) or 180 (HMS180) minutes. Rats separated from their mothers for prolonged periods (HMS180) display, as adults, abnormal anxiety-related behaviour as well as an exaggerated endocrine response to an acute stressor (McIntosh *et al*., 1999; Plotsky & Meaney, 1993; Ladd *et al*., 1996; Biagini *et al*., 1998; Wigger & Neumann, 1999, Ladd *et al*., 2000, 2004; Huot *et al*., 2001; Kalinichev *et al*., 2002).

It has been suggested that brain-derived neurotrophic factor (BDNF), a member of the nerve-growth factor family of neurotrophic factors, mediates some of the deleterious effects of stress in the hippocampus (Duman *et al*., 1997; Duman & Monteggia, 2006). Recent studies have shown that stress can decrease BDNF mRNA expression in the hippocampus, neocortex and amygdala, and increase it in others areas, e.g. hypothalamus (Smith *et al*., 1995a,b; Russo-Neustadt *et al*., 2001). Studies examining BDNF mRNA levels in HMS180 rats show increased expression in the prefrontal cortex and hippocampus of PND17 rats (Cirulli *et al*., 2003; Roceri *et al*., 2004) and decreased expression in the prefrontal cortex of adults (Roceri *et al*., 2004). BDNF protein levels in maternally stressed rats have only been measured by ELISA assays, which does not distinguish between the precursor (pro-BDNF) and mature forms of BDNF. In HMS180 rats, BDNF concentrations were unchanged or increased in the hippocampus (Roceri *et al*., 2004; Greisen *et al*., 2005), decreased in the striatum (Roceri *et al*., 2004) and unchanged in the frontal cortex and hypothalamus (Greisen *et al*., 2005). Recent studies suggest that precursor and mature forms of BDNF, both of which are biologically active, interact with different receptors and have contrasting effects on neuronal plasticity (Lee *et al*., 2001; Pang *et al*., 2004).

The potential roles of the transcription factors cAMP response element binding protein (CREB) and ΔFosB in various brain regions were also investigated. Impaired levels of CREB, indicative of impaired CREB function, in the hippocampus have been observed following chronic stress (Laifenfeld *et al*., 2005). In contrast, increased CREB function in the nucleus accumbens causes an aversive emotional state and may reduce an animal's sensitivity to external emotional stimuli (Barrot *et al*., 2002). CREB is also an important regulator of BDNF expression in the brain, as CREB binds to the BDNF promoter and regulates BDNF transcription (Finkbeiner *et al*., 1997). ΔFosB, an isoform of FosB, is one of the most stable proteins expressed in the brain (Chen *et al*., 1997). It has been suggested that ΔFosB mediates long-term changes in striatal and

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cortical function following chronic perturbations such as addiction (Kelz & Nestler, 2000) or disease states such as Parkinson's disease (Tekumalla *et al*., 2001).

In summary, the present study investigated whether chronic maternal separation induces long-term behavioural and molecular alterations in adult animals. Locomotor activity and startle responses were recorded, and plasma hypothalamus–pituitary–adrenal (HPA) axis hormone concentrations, and expression levels of mature and pro-BDNF, as well as CREB and ΔFosB, by Western blot analysis were measured in several brain regions of adult animals maternally separated postnatally compared to normal animal facility-reared (AFR) controls.

Materials and methods

Generation of maternal-separated rats

Detailed animal care and rearing protocols, previously described in detail by Huot *et al*. (2001), were used with slight modifications. Briefly, outbred, timed-pregnant Long–Evans hooded rats [Crl:(LE)BR; Charles River, Portage, MI, USA] arrived at our animal facility on gestational day 12. Pregnant dams were house in individual polycarbonate cages on a 12 : 12 h light : dark cycle (lights on at 08.00 h) with food and water freely available. On PND2, each litter was removed from their home cage and culled to six male and two female pups per dam. From PND2 to 14, each dam was transferred to an adjacent cage and the litter then exposed to one of three rearing conditions once daily: (i) AFR, which comprised home-cage bedding material changes and brief handling twice weekly beginning on PND5, with no other handling or separation; (ii) handled animals which were removed from the home cage daily for 15-min periods (HMS15); and (iii) maternal separation in which pups were removed from the home cage for 180 min daily (HMS180). During separations, litters were each in a small container with nesting material. These containers were placed in an incubator maintained at 32 ± 0.5 °C from PND2 to 5, and 30 ± 0.5 °C from PND6 to 14. This, along with huddling behaviour, minimized any body temperature loss during separation. At the end of the separation period, pups were returned to their home cage and reunited with their dam. These manipulations were initiated between 08.00 and 16.00 h in a semirandomized order. Approximately half of the cage bedding material was changed once per week beginning on PND5 when the dam and pups were out of the home cage. Litters were weaned on PND21, marked with identification codes and placed into the same rearing groups of six male animals per cage until PND30, at which time they were pair-housed. For all behavioural, endocrine and molecular experiments, only male rats were used. All experiments were performed with approval by the Emory University IACUC and complied with Federal requirements (NIH Guidelines for the Care and Use of Laboratory Animals). Data are presented as mean ± SEM and significance was $P < 0.05$.

Locomotor testing

General locomotor activity and stereotypy-like behaviour were measured using the Truscan Photobeam Activity system (Coulbourn Instruments, Allentown, PA, USA) when animals were between PND50 and 60. Rats were individually placed into an activity arena (41 × 41 × 41 cm) for a 30-min test. Measures included horizontal plane movement (total and in 5-min time bins), instances of vertical plane movement (rearing), and time spent grooming.

Acoustic startle testing

Startle experiments were conducted following locomotor testing using commercial instrumentation (SR-LAB, San Diego Instruments, San Diego, CA, USA) once male offspring were between PND50 and 60. Each of the four startle chambers (length, 16 cm; diameter, 8.2 cm) was housed within a ventilated, sound-isolation box with constant 72 dB ambient noise. Acoustic stimulus intensities and response intensities were calibrated (SR-LAB Startle Calibration System) to be nearly identical in each of the chambers. A piezoelectric accelerometer affixed to the animal enclosure frame was used to detect and transduce motion resulting from each animal's response; this was then recorded by a computerized data acquisition and analysis system. Rats were habituated to room transfer and the startle chambers for 4 days prior to actual startle presentation. Acoustic startle trials employed the following parameters: 50-ms sound pulses at 95, 110 or 125 dB with a 30-s intertrial interval and 10 repetitions per trial. A measure of startle response amplitude was derived from the mean of 100 digitized data points collected from stimulus onset at a rate of 1 kHz.

Catheterization and blood collection

The jugular vein of each adult (> PND60) rat was catheterized 4 days prior to airpuff startle testing and blood sampling as previously described by Thrivikraman *et al*. (2002), under anesthesia, with the use of aseptic surgical techniques as described in Thrivikraman *et al*. (2002); routine use of antibiotics is discouraged by our Department of Veterinary Resources and our IACUC at Emory University. The anaesthetic mixture routinely for our surgeries is composed of ketamine, xylazine and acepromazine (31.25, 6.25 and 1.25 mg/kg, respectively, subcutaneously). This 'balanced anaesthetic' provides muscle relaxation, loss of consciousness, and analgesia (Welberg *et al*., 2006). Following recovery from anaesthesia, rats were individually housed in testing cages located in the testing room. Food and water were freely available and the room was maintained on a 12 : 12 h light : dark cycle (lights on at 07.00 h). A 4-day postsurgical recovery period elapsed prior to testing with daily handling of each animal. On day 5 postsurgery, blood (0.300 mL/sample) was collected through the jugular cannula in awake, unrestrained rats into chilled 1.5-mL polyetheylene tubes containing 10 μL EDTA (100 mg/mL) at rest and at 5, 10, 15, 30, 45 and 60 min following airpuff startle (APS; see below). Blood samples were stored on ice until centrifugation (12 500 ***g***; 4 °C, 12 min), and the plasma separated and frozen (−20 °C) until assay.

APS testing

The mild stressor of APS was used to assess HPA axis function beginning at 08.30 h on PND65–75 animals. APS consisted of three blocks of three airpuffs (each puff ∼2 s) with 1 min between successive blocks (Engelmann *et al*., 1996). Each puff was directed toward the side of the head from a distance of ∼10 cm using a compressed air source (Dust-Off; Fisher, Atlanta, GA, USA).

Radioimmunoassays

The corticosterone (CORT) assay was performed in 5-μL plasma samples using the ImmuChem double antibody kit (ICN Biomedicals, Costa Mesa, CA, USA) with 100 μL trace and 100 μL antibody (Thrivikraman *et al*., 2000). The working range for CORT was

3–1000 ng/mL with <8% intra-assay coefficients of variation for either assay. Adrenocorticotropin (ACTH) was assayed in 100-μL plasma samples using the Allegro HS-ACTH kit (Nichols Institute, San Juan Capistrano, CA, USA) with 50 μL of iodinated tracer and one avidin-coated bead per tube as previously described (Thrivikraman *et al*., 2000). Sensitivity was 1 pg/tube with an EC_{50} of 15 pg and working range 1–1500 pg/mL.

Statistical analysis

Data were analysed with repeated-measures ANOVA using SAS software to determine statistical significance ($P < 0.05$). Data are presented as mean ± SEM.

Western blot analysis

Animals were killed on or after PND95 by decapitation; the brain was rapidly removed and immediately frozen on dry ice. The frozen brains were cut into 2- to 3-mm-thick coronal slices and brain punches taken for the appropriate region. Individual animal samples were coded by colour to three different groups to reflect the housing condition of the animal, to ensure that the person performing the Western blots was blind to the housing condition of the animal. Western blot analysis was similar to that previously described (Gemelli *et al*., 2006). Briefly, brain regions were isolated and homogenized in lysis buffer (Hepes, 25 mM; NaCl, 150 mM; EDTA, 2 mM; DTT, 1 mM; NP-40, 0.1%; aprotinin, 1 μM; leupeptin, 1 μM; NaF, 10 mM; and 1 × protease inhibitor) and protein concentration determined by the modified Lowry method (Biorad, Hercules, CA, USA). Samples of total protein (30 μg) in reducing dye were boiled for 3 min then electrophoresed on a 12% SDS-polyacrylamide gel for BDNF and an 8% SDS-polyacrylamide gel for CREB and ΔFosB. Each individual sample was electrophoresed on polyacrylamide gels such that all three colour groupings were represented on each gel. Proteins were transferred to nitrocellulose membrane then blocked in 1 × TBS with 0.1% Tween 20 and 10% nonfat dry milk for 1 h at room temperature (RT). The blots were then incubated in the presence of the rabbit anti-BDNF (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CREB (1 : 1000; Upstate, Lake Placid, NY, USA), rabbit ΔFosB (1 : 1000; Santa Cruz), or mouse antiactin (1 : 2000; ICN, Santa Cruz) in fresh blocking solution overnight at 4 °C. The blots were washed three times for 10 min in 1 × TBS with 0.1% Tween 20 at RT and then incubated for 1.5 h at RT with a peroxidase-labelled goat antirabbit IgG (1 : 2000; Vector Laboratories, Burlingame, CA, USA) or goat antimouse IgG (1 : 10 000; Vector Laboratories) then washed four times for 10 min in 1 × TBS with 0.1% Tween 20 followed by a final wash in 1 × TBS. Bands were visualized using enhanced chemiluminesescence (Amersham, Piscataway, NJ, USA). The density of the individual bands was quantified with the Scion Image Program and then averaged by colour groups; results are expressed as mean percentages of control values (± SEM). Only after the values for the individual groups were averaged for all experiments was the colour code revealed.

Results

Rat pups were maternally separated daily on PNDs2–14 for either 15 (HMS15) or 180 (HMS180) minutes and then assessed for long-term alterations in locomotor activity (ambulation) as adults. The HMS180 animals were significantly hypoactive compared to the briefly separated HMS15 or AFR rats in a 30-min trial (Fig. 1A, insert; $P < 0.05$). To gain a better understanding of this difference, the data were analysed in 5-min epochs (Fig. 1A): there were significant differences among groups ($F_{2,22} = 27.64$, $P < 0.0001$), the number of beam breaks significantly decreased over time ($F_{5,55} = 231.74$, $P < 0.0001$), with a significant interaction between group and time ($F_{10,110} = 2.75$, $P < 0.0046$). The HMS180 rats were significantly hypoactive compared to the AFR animals for the first 20 min of the 30-min test and then were indistinguishable in terms of locomotor activity from these controls. During this 30-min period in the locomotor boxes, the number of rearings and groomings of each animal was also recorded. The HMS180 rats had a significant increase in the total number of rearing and grooming counts, more similar to stereotypic behaviour, than the HMS15 or AFR rats (Fig. 1B and C). For rearing counts, there were significant differences among groups ($F_{2,22} = 35.92$, $P < 0.0001$), the number of rearing counts significantly decreased over time ($F_{5,55} = 80.25$, $P < 0.0001$) and there was a significant interaction between group and time ($F_{10,110} = 14.58$, $P < 0.001$). For grooming counts, there were significant differences among groups ($F_{2,22} = 27.88$, $P < 0.0001$), the amount of time spent grooming significantly decreased over time ($F_{5,55} = 95.98$, $P < 0.0001$) and there was a significant interaction between group and time ($F_{10,110} = 13.38$, $P < 0.0001$). Interestingly, the significant increases in number of rearing counts and time spent grooming of the HMS180 rats were restricted to the initial 10 min of the test, though the significant hypoactivity persisted for 20 min, suggesting that the hypoactivity was not simply the result of the increased rearing or grooming counts.

We also examined the responses of the maternally separated rats following acoustic startle (Fig. 2). We found that there was a significant difference among groups ($F_{2,22} = 5.33$, $P < 0.0129$) in response to startle amplitude ($F_{2,22} = 194.6$, $P < 0.0001$). At 95 and 110 dB, the HMS180 rats were indistinguishable from the HMS15 and AFR animals. However, at the 125 dB amplitude the HMS180 rats displayed a significantly heightened response (26% increase) compared to the other two sets of animals.

Maternally separated rats exhibited increased plasma concentrations of CORT and ACTH following acute stress

To assess HPA axis function in the maternally separated rats, we measured plasma CORT and ACTH concentrations following airpuff startle. Basal plasma CORT concentrations were similar among the three groups (AFR, 19.89 ± 1.36 ng/mL; HMS15, 21.39 ± 1.31; HMS180, 21.56 ± 1.33). Following airpuff startle, there was a significant rearing-associated ($F_{2,22} = 1223$, $P < 0.0001$) increase in plasma CORT concentrations over time in HMS180 animals ($F_{6,66} = 562.38$, $P < 0.0001$). The plasma CORT concentrations were significantly elevated within 10 min of airpuff startle in the HMS180 group and remained significantly higher for at least 60 min compared to the other two groups (Fig. 3A). We also found a significant rearing-associated ($F_{2,22} = 338.19$, $P < 0.0001$) increase in plasma ACTH concentrations over time ($F_{6,66} = 381.68$, $P < 0.0001$) following airpuff startle. Plasma ACTH concentrations were significantly increased in the HMS180 rats compared to HMS15 and AFR animals within 5 min following airpuff startle, and remained elevated for at least 60 min although the levels were starting to return to baseline (Fig. 3B). Similar to the CORT finding, plasma ACTH concentrations were unchanged in the HMS180 rats compared to the other two groups at resting conditions (AFR, 13.74 ± 1.42 pg/mL; HMS15, 11.84 ± 1.10; HMS180, 12.50 ± 1.08).

Maternally separated rats showed differential regulation of mature and immature BDNF protein expression in brain

Western blot analysis was used to measure levels of BDNF expression in the hippocampus, cortex, ventral tegmental area, nucleus accumbens, striatum and amygdala. The amount of BDNF protein was analysed as a ratio between BDNF and actin, the control protein, to ensure that an equal amount of protein was loaded onto the gel and transferred to the membrane. The Western blot data revealed a significant reduction in BDNF mature protein in the hippocampus (67% of AFR levels) and striatum (78%) of HMS180 rats (Fig. 4A; $P < 0.05$). In contrast, we found a significant increase in mature BDNF levels in the ventral tegmental area (136%) of HMS180 rats compared to AFR controls (Fig. 4A; $P < 0.05$). No significant change in mature BDNF protein levels was observed in the cortex, nucleus accumbens or amygdala of the HMS180 animals. The mature BDNF levels from the HMS15 samples in all brain regions examined were not significantly different from those of AFR animals.

We also examined whether neonatal maternal separation produced alterations in the precursor form of BDNF (pro-BDNF). Western blot analysis was able to differentiate between the mature (14 kDa) and the immature (32 kDa) forms of BDNF. The levels of immature BDNF protein were analysed as a ratio with actin. Pro-BDNF concentrations were significantly increased in the ventral tegmental area (141%) of HMS180 rats compared to AFR animals (Fig. 4B). Surprisingly, no significant change in pro-BDNF levels in the hippocampus or striatum of HMS180 animals was observed in spite of significant decreases in the mature form in these brain regions. No significant change in pro-BDNF levels was observed in the cortex, nucleus accumbens or amygdala of the HMS180 rats compared to AFR rats. The pro-BDNF

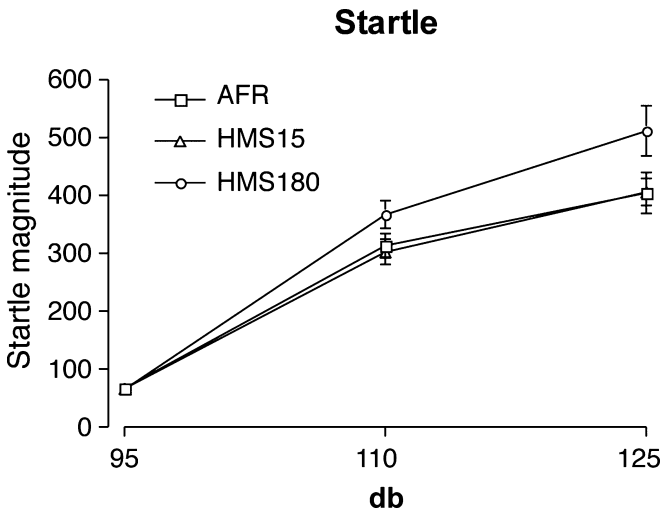

FIG. 2. Following acoustic startle, there was a significant rearing-associated ($F_{2,22} = 5.33$, $P = 0.0129$) increase in response to startle amplitude ($F_{2,22} = 194.6$, $P < 0.0001$). The HMS180 group had a significant increase in response to startle amplitude vs. the control groups at 125 dB. The ARF and HMS15 animals were indistinguishable from one another in their response to startle at each of the three startle amplitudes tested. Each group consisted of 12 adult animals that were PND75–90. Values are means ± SEM.

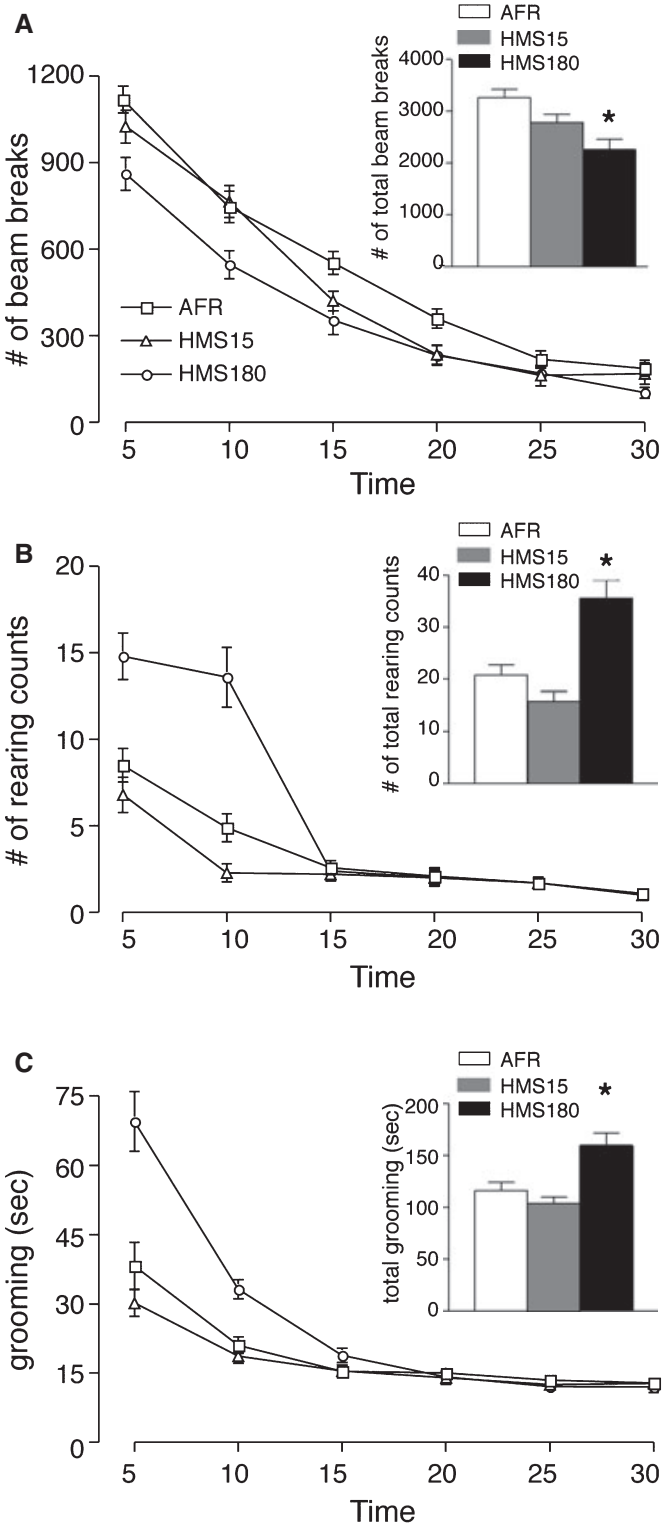

FIG. 1. (A) There were significant differences in locomotor activity among groups ($F_{2,22} = 27.64$, $P < 0.0001$) with a significant interaction between group and time ($F_{10,110} = 2.75$, p = 0.0046). (B) The average number of total beam breaks (ambulation) was significantly different between the HMS180 and the control groups (insert). There were significant differences in number of rearing counts among groups ($F_{2,22} = 35.92$, $P < 0.0001$) with a significant interaction between group and time ($F_{10,110} = 14.58$, $P < 0.0001$). The average number of total rearing counts during the 3-min test was significantly augmented in the HMS180 animals compared to the other two groups (insert). (C) There were significant differences in time spent grooming among groups ($F_{2,22} = 27.88$, $P < 0.0001$) with a significant interaction between group and time ($F_{10,110} = 13.38$, p = $P < 0.0001$). The average number of total rearing counts was also significantly increased in the HMS180 animals compared to the AFR and HMS15 rats (insert). Each group consisted of 12 adult animals that were PND75–90. Values are means ± SEM. *$P < 0.05$.

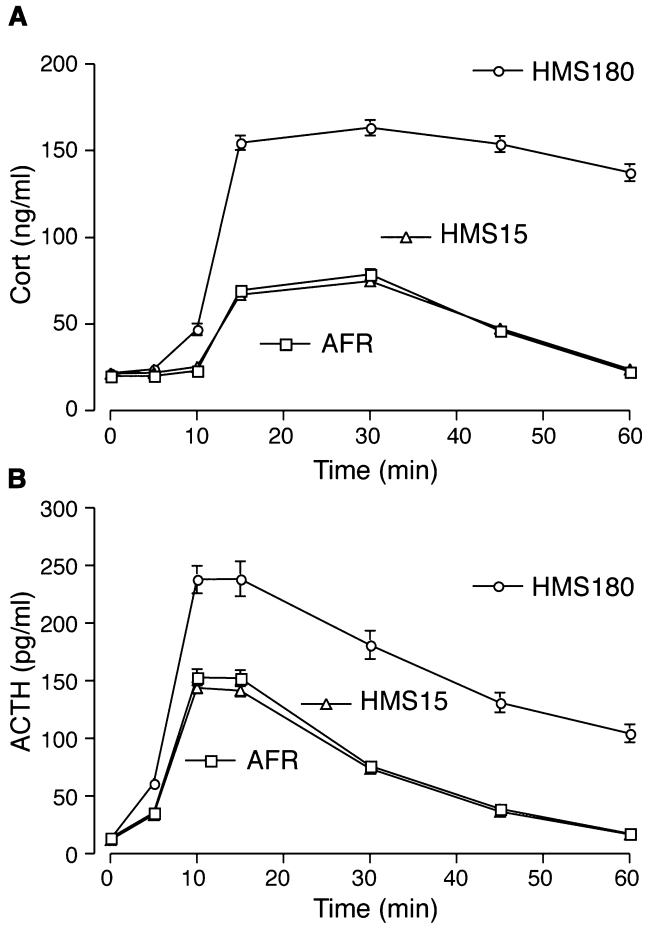

FIG. 3. (A) Corticosterone levels in the blood following airpuff startle were significantly elevated in response to rearing condition ($F_{2,22} = 1223.01$, $P < 0.0001$) over time ($F_{6,66} = 562.38$, $P < 0.0001$) with a significant interaction between group and time ($F_{12,132} = 118.68$, $P < 0.0001$). (B) Following airpuff startle, significant elevations in plasma ACTH levels were associated with rearing condition ($F_{2,22} = 338.19$, $P < 0.0001$) over time ($F_{6,66} = 381.68$, $P < 0.0001$) with a significant interaction between group and time ($F_{12,132} = 16.00$, $P < 0.0001$). There were no rearing-associated differences between basal plasma CORT or ACTH concentrations. Each group consisted of 12 adult animals that were PND75–90. Values are means ± SEM.

levels from the HMS15 samples in all brain regions examined were not significantly different from those of AFR animals.

CREB and ΔFosB levels were unaltered in maternal-separated rats

Levels of the transcription factors CREB and ΔFosB were analysed relative to actin with Western blot analysis. No significant differences in CREB levels were observed between HMS180, HMS15 and AFR animals in the hippocampus, cortex, ventral tegmental area, nucleus accumbens, striatum, or amygdala (Table 1). Moreover, levels of ΔFosB in cortex, striatum and nucleus accumbens of HMS180 rats were indistinguishable from those of the other two groups (Table 2).

Discussion

The present results show that rats exposed to maternal separation display, as adults, behavioural deficits, abnormal HPA axis function in response to an acute stressor, and long-term alterations in BDNF protein levels. Specifically, the HMS180 rats exhibited decreased activity, increased stereotypic behaviour and increased responses to acoustic startle compared with HMS15 and AFR rats. These HMS180 rats also displayed a heightened response to an acute stressor as demonstrated by their elevated plasma concentrations of CORT and ACTH in response to acoustic startle. Chronic maternal separation also resulted in abnormal levels of mature and immature BDNF in certain brain regions, suggesting that the changes observed are the result of specific long-term CNS alterations and not the result of generalized global deficits.

Postnatal maternal separation decreased locomotor activity but increased stereotypic behaviour in adult rats. The alterations in stereotyped locomotor behaviour were assessed in an activity chamber, a novel environment in which the rat is placed in the centre of the arena and movement is recorded. The stereotypic behaviours observed included increased rearing and grooming, all of which occurred within the first 10 min of testing. It is interesting that these changes in stereotypic behaviour were restricted to this initial period of testing because this maternal separation paradigm has been shown to increase anxiety-like behaviour in adults (McIntosh *et al*., 1999; Wigger & Neumann, 1999; Huot *et al*., 2001; Kalinichev *et al*., 2002). The increased stereotypic behaviour cannot, however, fully account for the hypoactivity observed in these rats because the decreased locomotor behaviour extended past the initial 10 min of testing.

The significant decrease in locomotor activity in the HMS180 animals is provocative as alterations in activity have been described in some depressed patients. It is interesting that the HMS180 animals also showed an increase in the number of rearing counts as well as in the time spent grooming compared to controls; however, how this would directly relate to the subsequent appearance of depression-like behaviour remains unclear. The fact that HMS180 animals displayed a significant decrease in locomotor activity, and that these changes were independent of other behavioural differences observed, suggests that the hypoactivity is due to a decrease in activity. Taken together, these data suggest that chronic neonatal maternal separation produces persistent long-term behavioural alterations that may underlie aspects associated with the development of mood disorders.

Chronic maternal separation has been shown to produce other long-term behavioural alterations, including increased anxiety-like behaviour and an augmented response to acoustic startle, in adult rats (McIntosh *et al*., 1999; Wigger & Neumann, 1999; Caldji *et al*., 2000; Huot *et al*., 2001; Kalinichev *et al*., 2002); these are similar to what we observed. These chronic maternally separated rats also exhibit alterations in neuroendocrine function. Basal concentrations of corticotropin-releasing factor (CRF) are elevated in HMS rats, and plasma concentrations of CORT and ACTH are augmented in chronic maternal-separated rats following acute stress (Plotsky & Meaney, 1993; Huot *et al*., 2001). These findings are believed to be the result of dysregulation of the HPA axis, although the molecular changes that may be contributing to these long-term alterations have yet to be identified. We investigated HPA axis activity in our rats and found a similar dysregulation as has been previously reported (Plotsky & Meaney, 1993; Huot *et al*., 2001), verifying the authenticity of the maternal separation procedure.

Because recent data suggests that precursor BDNF (pro-BDNF) may be biologically active (Mowla *et al*., 2001), we examined whether neonatal maternal separation produced alterations in this form of BDNF. The decrease in mature BDNF hippocampal levels in the adult animals that were exposed to maternal separation at birth is consistent with previous work suggesting that chronic stress paradigms can produce persistent adaptations of hippocampal BDNF (Smith *et al*.,

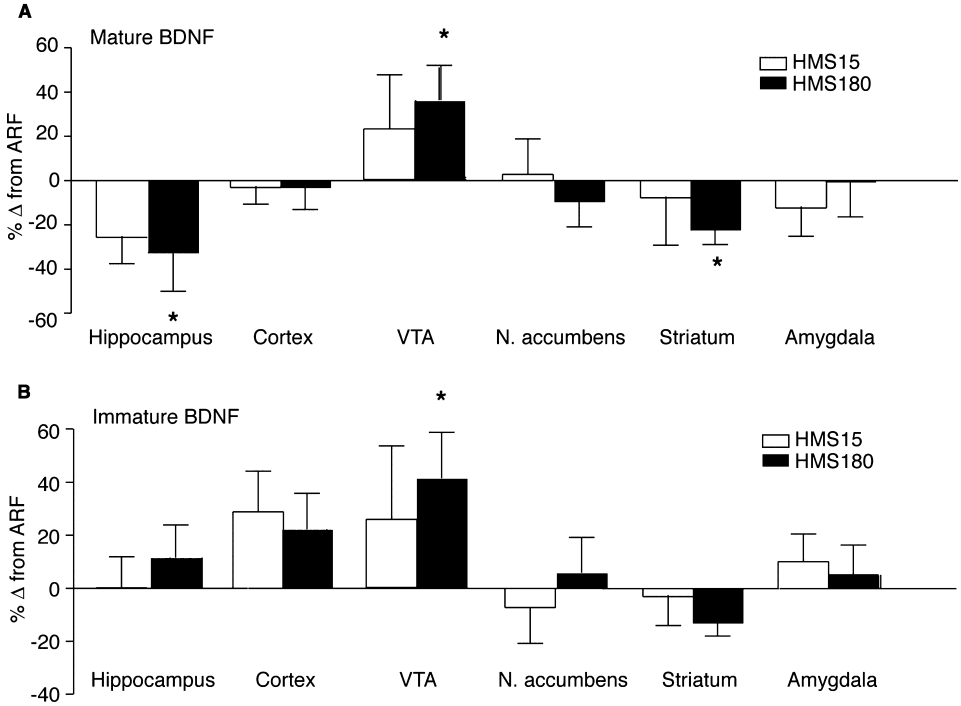

FIG. 4. (A) Levels of mature BDNF in the hippocampus were significantly reduced in HMS180 animals (33%, $P < 0.05$) compared to ARF rats, with a trend towards a reduction in HMS15 rats (22%, $P > 0.05$). Mature BDNF expression in the cortex was unchanged in HMS15 (3%) and HMS180 (3%) animals. In the VTA, there was a nonsignificant trend towards increased mature BDNF levels in HMS15 rats (increased 25% compared to ARF animals; $P > 0.05$) and a significant increase in the HMS180 group (41%, $P < 0.05$). No significant change in mature BDNF levels were found in the nucleus accumbens for the HMS15 (3% increase) or HMS180 (8% decrease) groups compared to ARF animals. In the striatum there was no significant change in the HMS15 group (8% decrease) but there was a significant decrease in the HMS180 group (22%, $P < 0.05$). No significant change in mature BDNF levels was observed in amygdala in the HMS15 (11% decrease) or HMS180 (1% decrease) groups. Bars represent mean values ± SEM. (B) The hippocampus showed no significant change in immature BDNF in the HMS15 animals (100% of ARF values) and HMS180 rats (11% increase). In the cortex, there was a strong trend towards an increase in the HMS15 acute group (28%) and HMS180 group (22%), although it did not reach significance. In the VTA, we found a trend towards an increase in pro-BDNF expression in the HMS15 group (26%) and a significant increase in the HMS180 animals (41%, $P < 0.05$). Levels of pro-BNDF were not significantly altered in the nucleus accumbens in the HMS15 (7% decrease) or the HMS180 animals (6% increase). In the striatum, no significant change in pro-BDNF expression was observed in the HMS15 group (3% decrease) or the HMS180 animals (13% decrease). Pro-BDNF expression was also not significantly altered in the amygdala of HMS15 (10% increase) or HMS180 animals (5% increase) compared to ARF rats. Western blots for each region consisted of 9–12 adult animals/handled group that were >PND95. Bars represent mean values ± SEM. *Denotes statistical significance ($P < 0.05$.)

1995b). In the neonatal maternal separation paradigm, the changes in BDNF expression persisted for several weeks and may have contributed to long-term functional alterations of the hippocampus. Indeed, reduction in BDNF levels in the hippocampus by transgenic and other molecular approaches results in functional alterations including impairments in long-term potentiation and learning and memory (Korte *et al*., 1996; Patterson *et al*., 1996; Monteggia *et al*., 2004). Chronic neonatal maternal separation has been shown to produce significant impairments in hippocampal learning and memory as assessed in the Morris water maze (Huot *et al*., 2002). Furthermore, it has been suggested that decreased expression of BDNF in the hippocampus underlies depression-like behaviour (Duman & Monteggia, 2006). This long-lasting alteration in BDNF levels produced by maternal separation may contribute to functional changes in neuronal plasticity as well as underlie some of the 'depression-like' behaviour, including the alterations in locomotor activity and stereotypic behaviour, observed in the maternally separated rats.

This decrease in hippocampal BDNF expression is in contrast to recent findings suggesting that BDNF levels are either unaltered (Roceri *et al*., 2004) or increased (Greisen *et al*., 2005) in this brain region of animals undergoing a similar maternal separation paradigm as neonates. The discrepancy may be due to the fact that we examined the levels of BDNF by Western blot analysis to differentiate the mature and pro-BDNF levels instead of measuring BDNF release by ELISA assays, which utilize an antibody that would not differentiate these two forms.

Alterations in the expression level of either pro-BDNF or mature BDNF may contribute to specific alterations in brain function. Pro-BDNF is proteolytically cleaved by tPA-plasmin to yield mature BDNF (Lee *et al*., 2001; Chen *et al*., 2004; Pang *et al*., 2004). However, recent studies suggest that pro-BDNF has distinct and somewhat opposite effect to mature BDNF on cell survival and synaptic plasticity. It has been suggested that increased levels of pro-BDNF induce apoptosis and long-term depression in the hippocampus while increased levels of mature BDNF are associated with increased cell survival and long-term potentiation (Teng *et al*., 2005; Ghosh *et al*., 1994; Korte *et al*., 1998; Woo *et al*., 2005). The selective decrease in mature BDNF in the hippocampus of chronic maternally separated animals may point to decreased cell survival and synaptic function in the hippocampus, alterations that, it has been suggested, are involved in mediating depression-like behaviour (Duman & Monteggia, 2006).

A significant increase in levels of mature and immature BDNF in the ventral tegmental area (VTA) was also observed. This brain region contains dopamine neurons integral to reward. Many studies have shown that stress activates these VTA dopamine neurons and

TABLE 1. Protein levels of CREB in maternally separated rats

Region	*n*	CREB protein concentration (Percentage of AFR group) HMS15	HMS180
Hippocampus	12	83 ± 18	118 ± 24
Cortex	9	102 ± 9	86 ± 9
VTA	9	109 ± 6	98 ± 11
Nucleus accumbens	9	96 ± 7	95 ± 10
Striatum	9	92 ± 13	97 ± 10
Amygdala	9	95 ± 19	96 ± 17

TABLE 2. Protein levels of ΔFosB in maternally separated rats

Region	*n*	ΔFosB protein concentration (Percentage of AFR group) HMS15	HMS180
Cortex	12	88 ± 6	92 ± 9
Nucleus accumbens	12	97 ± 13	98 ± 9
Striatum	11	101 ± 8	95 ± 8

stimulates dopamine transmission in rats (Horger & Roth, 1996; Di Chiara *et al*., 1999; Yadid *et al*., 2001; Nieoullon & Coquerel, 2003). Recent work has shown that intra-VTA injections of BDNF produce an increase in 'depression-like' behaviour in rats as assessed in the forced swim test (Eisch *et al*., 2003). Our data suggest that chronic neonatal stress produces long-term changes of BDNF expression in the VTA, consistent with a phenotype that may include aspects of depression-like behaviour.

We also observed a significant decrease in mature BDNF levels in the striatum of adult rats that underwent chronic neonatal maternal separation, in agreement with a recent study showing that chronic maternal stress decreases BDNF levels in the striatum of adult rats (Roceri *et al*., 2004). It is interesting that we observed normal levels of mature and immature BDNF in the cortex of these chronic maternally separated adult animals because BDNF has been shown to be transported in an anterograde manner from the frontoparietal cortex to the striatum (Altar *et al*., 1997). However, it is important to note that our cortical dissections were from Fr2 and the dorsal anterior cingulate area, Cg1, which are within rat prefrontal cortex, so it is possible that we did not observe a change in BDNF levels due to the restricted localization of our dissections.

Although we observed altered levels of BDNF protein in several brain regions, we did not observe an alteration in the level of CREB protein. CREB is a transcription factor that regulates BDNF transcription in a phosphorylation-dependent manner in the brain. We measured total protein levels of CREB, and therefore we may have missed transient changes in phosphorylation–dephosphorylation levels of CREB, which are more relevant to its role as a transcription activator. In addition, recent reports have suggested that the transcription factor nuclear factor of activated T cells (NFAT) may be a more critical regulator of BDNF expression in the brain (Graef *et al*., 2003). Further studies are needed to examine whether chronic maternal separation regulates transcription factors such as NFAT and impacts on BDNF levels.

We also measured levels of ΔFosB because it has been suggested that this transcription factor mediates long-term functional alterations in brain, particularly in the striatum. No alteration in the level of ΔFosB in the striatum, nucleus accumbens or prefrontal cortex of adult animals exposed to chronic maternal separation as neonates was observed.

Our results suggest that chronic neonatal maternal separation induces long-term alterations in adult behaviour as well as in BDNF expression, in particular in processing of mature and pro-BDNF in the hippocampus, striatum and ventral tegmental area. Moreover, these changes are specific and not the result of global changes in protein expression as no long-term alterations in the levels of CREB, a transcriptional factor that can regulate BDNF transcription, or in ΔFosB levels were observed in any brain region analysed. The functional consequences of persistent alterations in mature and/or pro-BDNF levels in specific brain regions may be quite profound and contribute to the long-term behavioural phenotypes observed in the adult animals. These data start to provide a neural framework whereby early life stressors may contribute to persistent changes in gene expression that underlie the development of mood disorders.

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Abbreviations

ACTH, adrenocorticotropin; AFR, normal animal facility-reared; APS, airpuff startle; BDNF, brain-derived neurotrophic factor; CORT, corticosterone; CREB, cAMP response element binding protein; ΔFosB, an isoform of FosB; HMS15, maternal separation for 15 min; HMS180, maternal separation for 180 min; HPA, hypothalamus–pituitary–adrenal; PND, postnatal day; VTA, ventral tegmental area.

References

Altar, C.A., Cai, N., Bliven, T., Juhasz, M., Conner, J.M., Acheson, A.L., Lindsay, R.M. & Wiegand, S.J. (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature*, **389**, 856–860.

Arborelius, L., Hawks, B.W., Owens, M.J., Plotsky, P.M. & Nemeroff, C.B. (2004) Increased responsiveness of presumed 5-HT cells to citalopram in adult rats subjected to prolonged maternal separation relative to brief separation. *Psychopharmacology (Berl.)*, **176**, 248–255.

Barrot, M., Olivier, J.D., Perrotti, L.I., DiLeone, R.J., Berton, O., Eisch, A.J., Impey, S., Storm, D.R., Neve, R.L., Yin, J.C., Zachariou, V. & Nestler, E.J. (2002) CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl Acad. Sci. USA*, **99**, 11435–11440.

Biagini, G., Pich, E.M., Carani, C., Marrama, P. & Agnati, L.F. (1998) Postnatal maternal separation during the stress hyporesponsive period enhances the adrenocortical response to novelty in adult rats by affecting feedback regulation in the CA1 hippocampal field. *Int. J. Dev. Neurosci.*, **16**, 187–197.

Caldji, C., Francis, D., Sharma, S., Plotsky, P.M. & Meaney, M.J. (2000) The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology*, **22**, 219–229.

Chen, J., Kelz, M.B., Hope, B.T., Nakabeppu, Y. & Nestler, E.J. (1997) Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *J. Neurosci.*, **17**, 4933–4941.

Chen, Z.Y., Patel, P.D., Sant, G., Meng, C.X., Teng, K.K., Hempstead, B.L. & Lee, F.S. (2004) Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *J. Neurosci.*, **24**, 4401–4411.

Cirulli, F., Berry, A. & Alleva, E. (2003) Early disruption of the mother-infant relationship: effects on brain plasticity and implications for psychopathology. *Neurosci. Biobehav Rev.*, **27**, 73–82.

Di Chiara, G., Loddo, P. & Tanda, G. (1999) Reciprocal changes in prefrontal and limbic dopamine responsiveness to aversive and rewarding stimuli after chronic mild stress: implications for the psychobiology of depression. *Biol. Psychiatry*, **46**, 1624–1633.

Duman, R.S., Heninger, G.R. & Nestler, E.J. (1997) A molecular and cellular theory of depression. *Arch. Gen. Psychiatry*, **54**, 597–606.

Duman, R.S. & Monteggia, L.M. (2006) A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry*, **59**, 1116–1127.

Eisch, A.J., Bolanos, C.A., de Wit, J., Simonak, R.D., Pudiak, C.M., Barrot, M., Verhaagen, J. & Nestler, E.J. (2003) Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biol. Psychiatry*, **54**, 994–1005.

Engelmann, M., Thrivikraman, K.V., Su, Y., Nemeroff, C.B., Montkowski, A., Landgraf, R., Holsboer, F. & Plotsky, P.M. (1998) Endocrine and behavioral effects of airpuff-startle in rats. *Psychoneuroendocrinology*, **21**, 391–400.

Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M. & Greenberg, M.E. (1997) CREB: a major mediator of neuronal neurotrophin responses. *Neuron*, **19**, 1031–1047.

Gemelli, T., Berton, O., Nelson, E.D., Perrotti, L.I., Jaenisch, R. & Monteggia, L.M. (2006) Postnatal loss of methyl-CpG binding protein 2 in the forebrain is sufficient to mediate behavioral aspects of Rett syndrome in mice. *Biol. Psychiatry*, **59**, 468–476.

Ghosh, A., Carnahan, J. & Greenberg, M.E. (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. *Science*, **263**, 1618–1623.

Graef, I.A., Wang, F., Charron, F., Chen, L., Neilson, J., Tessier-Lavigne, M. & Crabtree, G.R. (2003) Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell*, **113**, 657–670.

Greisen, M.H., Altar, C.A., Bolwig, T.G., Whitehead, R. & Wortwein, G. (2005) Increased adult hippocampal brain-derived neurotrophic factor and normal levels of neurogenesis in maternal separation rats. *J. Neurosci. Res.*, **79**, 772–778.

Hall, F.S. (1998) Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. *Crit. Rev. Neurobiol.*, **12**, 129–162.

Horger, B.A. & Roth, R.H. (1996) The role of mesoprefrontal dopamine neurons in stress. *Crit. Rev. Neurobiol.*, **10**, 395–418.

Huot, R.L., Plotsky, P.M., Lenox, R.H. & McNamara, R.K. (2002) Neonatal maternal separation reduces hippocampal mossy fiber density in adult Long Evans rats. *Brain Res.*, **950**, 52–63.

Huot, R.L., Thrivikraman, K.V., Meaney, M.J. & Plotsky, P.M. (2001) Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. *Psychopharmacology (Berl.)*, **158**, 366–373.

Kalinichev, M., Easterling, K.W., Plotsky, P.M. & Holtzman, S.G. (2002) Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. *Pharmacol. Biochem. Behav.*, **73**, 131–140.

Kelz, M.B. & Nestler, E.J. (2000) deltaFosB: a molecular switch underlying long-term neural plasticity. *Curr. Opin. Neurol.*, **13**, 715–720.

Korte, M., Griesbeck, O., Gravel, C., Carroll, P., Staiger, V., Thoenen, H. & Bonhoeffer, T. (1996) Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice. *Proc. Natl Acad. Sci. USA*, **93**, 12547–12552.

Korte, M., Kang, H., Bonhoeffer, T. & Schuman, E. (1998) A role for BDNF in the late-phase of hippocampal long-term potentiation. *Neuropharmacology*, **37**, 553–559.

Ladd, C.O., Huot, R.L., Thrivikraman, K.V., Nemeroff, C.B., Meaney, M.J. & Plotsky, P.M. (2000) Long-term behavioral and neuroendocrine adaptations to adverse early experience. *Prog. Brain Res.*, **122**, 81–103.

Ladd, C.O., Huot, R.L., Thrivikraman, K.V., Nemeroff, C.B. & Plotsky, P.M. (2004) Long-term adaptations in glucocorticoid receptor and mineralocorticoid receptor mRNA and negative feedback on the hypothalamo-pituitary-adrenal axis following neonatal maternal separation. *Biol. Psychiatry*, **55**, 367–375.

Ladd, C.O., Owens, M.J. & Nemeroff, C.B. (1996) Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation. *Endocrinology*, **137**, 1212–1218.

Laifenfeld, D., Karry, R., Grauer, E., Klein, E. & Ben-Shachar, D. (2005) Antidepressants and prolonged stress in rats modulate CAM-L1, laminin, and pCREB, implicated in neuronal plasticity. *Neurobiol. Dis*, **20**, 432–441.

Lee, R., Kermani, P., Teng, K.K. & Hempstead, B.L. (2001) Regulation of cell survival by secreted proneurotrophins. *Science*, **294**, 1945–1948.

McIntosh, J., Anisman, H. & Merali, Z. (1999) Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. *Brain Res. Dev. Brain Res.*, **113**, 97–106.

Monteggia, L.M., Barrot, M., Powell, C.M., Berton, O., Galanis, V., Gemelli, T., Meuth, S., Nagy, A., Greene, R.W. & Nestler, E.J. (2004) Essential role of brain-derived neurotrophic factor in adult hippocampal function. *Proc. Natl Acad. Sci. USA*, **101**, 10827–10832.

Mowla, S.J., Farhadi, H.F., Pareek, S., Atwal, J.K., Morris, S.J., Seidah, N.G. & Murphy, R.A. (2001) Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. *J. Biol. Chem.*, **276**, 12660–12666.

Nieoullon, A. & Coquerel, A. (2003) Dopamine: a key regulator to adapt action, emotion, motivation and cognition. *Curr. Opin. Neurol.*, **16** (Suppl. 2), S3–S9.

Pang, P.T., Teng, H.K., Zaitsev, E., Woo, N.T., Sakata, K., Zhen, S., Teng, K.K., Yung, W.H., Hempstead, B.L. & Lu, B. (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science*, **306**, 487–491.

Patterson, S.L., Abel, T., Deuel, T.A., Martin, K.C., Rose, J.C. & Kandel, E.R. (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron*, **16**, 1137–1145.

Plotsky, P.M. & Meaney, M.J. (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Brain Res. Mol. Brain Res.*, **18**, 195–200.

Roceri, M., Cirulli, F., Pessina, C., Peretto, P., Racagni, G. & Riva, M.A. (2004) Postnatal repeated maternal deprivation produces age-dependent changes of brain-derived neurotrophic factor expression in selected rat brain regions. *Biol. Psychiatry*, **55**, 708–714.

Russo-Neustadt, A., Ha, T., Ramirez, R. & Kesslak, J.P. (2001) Physical activity-antidepressant treatment combination: impact on brain-derived neurotrophic factor and behavior in an animal model. *Behav. Brain Res.*, **120**, 87–95.

Smith, M.A., Makino, S., Kim, S.Y. & Kvetnansky, R. (1995a) Stress increases brain-derived neurotropic factor messenger ribonucleic acid in the hypothalamus and pituitary. *Endocrinology*, **136**, 3743–3750.

Smith, M.A., Makino, S., Kvetnansky, R. & Post, R.M. (1995b) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.*, **15**, 1768–1777.

Tekumalla, P.K., Calon, F., Rahman, Z., Birdi, S., Rajput, A.H., Hornykiewicz, O., Di Paolo, T., Bedard, P.J. & Nestler, E.J. (2001) Elevated levels of DeltaFosB and RGS9 in striatum in Parkinson's disease. *Biol. Psychiatry*, **50**, 813–816.

Teng, H.K., Teng, K.K., Lee, R., Wright, S., Tevar, S., Almeida, R.D., Kermani, P., Torkin, R., Chen, Z.Y., Lee, F.S., Kraemer, R.T., Nykjaer, A. & Hempstead, B.L. (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *J. Neurosci.*, **25**, 5455–5463.

Thrivikraman, K.V., Huot, R.L. & Plotsky, P.M. (2002) Jugular vein catheterization for repeated blood sampling in the unrestrained conscious rat. *Brain Res. Brain Res. Protoc.*, **10**, 84–94.

Thrivikraman, K.V., Nemeroff, C.B. & Plotsky, P.M. (2000) Sensitivity to glucocorticoid-mediated fast-feedback regulation of the hypothalamic-pituitary-adrenal axis is dependent upon stressor specific neurocircuitry. *Brain Res.*, **870**, 87–101.

Welberg, L.A., Kinkead, B., Thrivikraman, K., Huerkamp, M.J., Nemeroff, C.B. & Plotsky, P.M. (2006) Ketamine-xylazine-acepromazine anesthesia and postoperative recovery in rats. *J. Am. Assoc. Lab. Anim. Sci.*, **45**, 13–20.

Wigger, A. & Neumann, I.D. (1999) Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. *Physiol. Behav.*, **66**, 293–302.

Woo, N.H., Teng, H.K., Siao, C.J., Chiaruttini, C., Pang, P.T., Milner, T.A., Hempstead, B.L. & Lu, B. (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nature Neurosci.*, **8**, 1069–1077.

Yadid, G., Overstreet, D.H. & Zangen, A. (2001) Limbic dopaminergic adaptation to a stressful stimulus in a rat model of depression. *Brain Res.*, **896**, 43–47.