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Repeated neonatal separation results in different neurochemical and behavioral changes in adult male and female Mongolian gerbils

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Abstract

We assessed whether daily separation of Mongolian gerbils (*Meriones unguiculatus*) from mothers and siblings during postnatal days 4–20 would produce behavioral and neurochemical changes in adulthood that parallel some features of depression in humans. Neonatal separation altered the behavior of adult females in the open field test but not in the tail suspension test, and did not affect behavior of males. Separated males, but not females, showed a significant decrease in hippocampal brain derived neurotrophic factor (BDNF) relative to controls. Western blot and optical densitometry measurements in the hippocampus did not reveal significant group differences in synaptophysin levels in either sex, but there was a tendency toward decreased levels of synaptophysin in the entire hippocampus as well as the CA1 hippocampal subregion of separated males. Repeated separation of neonates from mothers and siblings led to subtle behavioral and neurochemical changes during adulthood that were expressed differently in male and female gerbils.

Keywords: Mongolian gerbil; Maternal separation; Open field test; Tail suspension test; Synaptophysin; Brain derived neurotrophic factor; Hippocampus; Substance P

1. Introduction

Early postnatal maternal and/or sibling separation in rodents is a frequently used model of an early-life stressor, which in humans has been correlated with the development of depression in later life (O'Connor and Cameron, 2006). In rodents, the manipulation has been reported to induce lasting neurochemical changes and to alter behavior, especially in response to stressful situations (Gilmer and McKinney, 2003; Heim et al., 2004). Although many studies using postnatal separation in rats (Huot et al., 2001; Zimmerberg et al., 2003) have reported behavioral characteristics in adulthood that resemble features of human depression and anxiety, others have not (Pryce et al., 2001, 2003).

Several neurochemical and anatomical differences that have been implicated in depression or its treatment might be affected by early social isolation. Neuroimaging and post-mortem studies of depressed individuals have described brain changes suggestive of decreased cell proliferation and integrity: reduced cortical thickness; loss of glia; decreased neuronal size; abnormal synaptic terminal arborization in the prefrontal cortex, hippocampus and amygdala; as well as reduced hippocampal volume (Benninghoff et al., 2002; Fossati et al., 2004; Rajowska, 2000). Some antidepressant therapies appear to attenuate or even reverse these changes (Hajszan et al., 2005; Malberg et al., 2000; Santarelli et al., 2003). Studies of stress-based animal depression models have reported structural and functional brain changes that resemble those seen in depressed humans (Czeh et al., 2001; Gamaro et al., 2003). Thus, there is support for the hypothesis that depression may involve structural and functional abnormalities in brain networks involved in the control of cognition and emotion and a weakened capacity to respond with appropriate plastic changes to stressors and other functional challenges (Fossati et al., 2004).

Neurotrophins play a crucial role in neuronal survival and differentiation and mediate both synaptic plasticity and

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morphological maintenance (Coyle and Duman, 2003). One neurotrophin in particular, brain derived neurotrophic factor (BDNF), has been reported to be altered in depression (Russo-Neustadt and Chen, 2005). Stress-related models of depression have also been associated with decreased BDNF protein and mRNA levels in the brain, especially in the hippocampus (Duman and Monteggia, 2006). Chronic treatment with antidepressants from several classes has been shown to increase expression of BDNF mRNA, and dose-dependently alter BDNF protein immunoreactivity in the rat hippocampus (Altieri et al., 2004). The link between BDNF and depression, its modulation by antidepressant treatments, ands its role in brain plasticity support the idea that alterations in neural plasticity may characterize depressed states.

Due to the rapid development of the rodent brain during the neonatal period, loss of normal stimulation from the mother and littermates may alter the dynamics of neurotrophin regulation in the brain and affect plasticity in adulthood. Roceri et al. (2002) reported a significant reduction in BDNF mRNA and protein in rat hippocampus after a single 24 h period of maternal and sibling separation on postnatal day (PND) 9, which manifested only in adulthood.

Neonatal separation has also been reported to significantly reduce synaptophysin immunoreactivity in the CA1 and CA3 hippocampal regions of adult rats (Andersen and Teicher, 2004). Synaptophysin is a presynaptic vesicle protein partially regulated by BDNF, that is commonly used as a marker of synaptic density and, by extension, of neural plasticity (Tartaglia et al., 2001). Modulation of synaptophysin expression levels in animal depression models appears to be region-specific, with some areas showing increases and others decreases in the protein (Conrad et al., 1999; Gao et al., 2006; Magariños and McEwen, 1995). Thus, measurement techniques that sample brain regions in different ways could yield different results. One aim of this study was to compare synaptophysin immunoreactivity measurements using two different optical densitometry (OD) sampling techniques.

Rodent studies suggest that drugs affecting Substance P (SP) and its neurokinin (NK) receptors, especially the NK_1 receptor, have potential antidepressant properties (Kramer et al., 1998; Rupniak et al., 2000; Salome et al., 2006; Santarelli et al., 2001). Despite mixed results from clinical investigations into the antidepressant effects of SP receptor antagonists (SPAs) (Keller et al., 2006; Kramer et al., 1998), there is continued interest in the development of such drugs for treating depression and/or anxiety (Holden, 2003). The use of rat and mouse models is limited with respect to such drug development because the affinity of NK receptors in these species differs from that of humans; Mongolian gerbils (*Meriones unguiculatus*), however, have NK receptors with an affinity similar to that of humans (Gitter et al., 1991). It would therefore be useful to develop behavioral and neurochemical models of depression in this species for further assessment of the effects of SP-related drugs.

This study assessed the effects of postnatal separation in Mongolian gerbils in order to facilitate use of this species in studying the antidepressant or anxiolytic roles of SPAs. It used two behavioral assays, the open field test (OFT) and tail suspension test (TST), and examined levels of BDNF and synaptophysin protein in the hippocampus of gerbils of both sexes following repeated, early separation from mother and siblings.

2. Materials and methods

2.1. Animals

Timed-pregnant Mongolian gerbils were purchased from Charles River Laboratories (Montréal, QC, Canada). Animals were individually housed in standard polypropylene cages and maintained in a temperature- and humidity-controlled room on a 12:12 LD cycle (lights on 0800 h). Laboratory rodent chow and water were available *ad libitum*. Apart from regular animal husbandry, gerbils were left undisturbed until after parturition, which was designated as PND 0. All procedures were approved by the Dalhousie University Committee on Laboratory Animals and were conducted in accordance with the Canadian Council on Animal Care guidelines.

2.2. Postnatal treatment

On PND 2, dams were temporarily removed from their home cages and placed individually into clean ones. Their litters were moved temporarily from the colony room into an adjacent room, where pups were sexed and new litters created. As often as possible, a pair of male and female pups was taken from each litter to create a new one. These new litters were placed into the foster dam's home cage and covered with shavings after which dams were returned. Following this fostering procedure, each dam had, at most, two genetic offspring of each sex and all litters consisted of 6–7 pups with 2–3 males and 3–5 females. On PND 4, litters were randomly assigned to the separation (S) or control (C) conditions. C litters remained with the dam and were undisturbed until weaning, apart from weighing on PND 20 as well as normal husbandry procedures, which included exchanging a portion of soiled shavings for clean shavings twice weekly. S litters were removed from the colony room to an adjacent room at 1300 h during PND 4–20. Individual pups were weighed and kept alone in polypropylene cages warmed with heating pads (~30 °C) for 4 h daily (1300–1700 h), and then returned to their home cages. All pups were weaned on PND 28 and then group-housed with same-sex littermates. A total of 78 S pups (12 litters) and 68 C pups (11 litters) were studied.

2.3. Treatment during adulthood

Animals were weighed and housed individually on PND 56. Daily handling started on PND 70 and continued until PND 80. Handling consisted of weighing, holding and stroking the animals, as well as taking them out of the colony room into an adjacent testing room; 2–3 min was spent with each animal. Subsequently, animals were assigned to be studied in one of three ways: behaviorally, using Western blotting, or using immunohistochemistry (IHC). One animal of each sex from each litter was assigned randomly to be studied behaviorally or using Western blotting; the remaining animals were used in IHC studies.

To assess estrous cycles, females underwent vaginal swabbing daily at 0900–1000 h once they had become accustomed to handling. Vaginal smears were applied to glass slides, fixed in ethanol, rinsed in dH_2O, and stained in a 20% Giemsa (Sigma–Aldrich, St Louis, MO, USA) solution for 30 min. Stage of estrous cycle was determined according to established criteria by viewing dried slides under a light microscope (Nishino and Totsukawa, 1996). Females were tested behaviorally or killed for neurochemical studies only when they were in the estrous stage.

2.4. Open field and tail suspension tests

Following handling, all males and estrous females undergoing behavioral testing were studied using the open field test (OFT). The open field was a lidless wooden box (72 × 72 × 36 cm) with a plexiglas floor overlaying a 25-square grid. Gerbils were placed individually in one corner of the field, and their activity was video-recorded for 5 min. Number of grid line-crosses, rearing and grooming activities and incidents of seizure-like episodes were scored, as was the time spent in the central portion of the field, defined by the inner nine squares. An animal was considered to be in the central portion when both rear paws were in the inner squares.

Approximately 24 h after the OFT, animals were tested on the tail suspension test (TST). The gerbil's tail was taped 1 cm from the base onto a flat metal strip that was clamped to a rotarod stand, positioning the gerbil ~0.5 m above the floor. The test was video-recorded for 6 min and duration of immobility was scored. Immobility was defined as the absence of body movements except those required for respiration. Both tests were conducted in a room with fluorescent lighting (~300 lx) between 1300 and 1500 h. Because the TST was conducted 24 h after the OFT, females were tested regardless of their estrous cycle stage. Animals that exhibited seizures during testing were excluded from scoring. Behavior was scored by a trained observer, blind to the sex and treatment history of the animal.

For all scored variables, averages and standard errors of the mean (SEM) were calculated for each group (S females, S males, C females, C males). All behavioral data were analyzed with two-factor ANOVAs, with sex and postnatal condition (S and C) as the two factors; these were followed by Bonferroni *post hoc* tests (SPSS, Version 13.0 for Windows; GraphPad Prism 4.0c for Macintosh). A similar analysis was conducted to compare behaviors between the first and last minutes of the 5 min OFT, using $\alpha < 0.05$ as the criterion for statistical significance.

2.5. Immunohistochemistry

Following anesthetic overdose, IHC group animals were perfused transcardially with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains were extracted, post-fixed at 4 °C in 4% PBS, and cut on a vibratome in 40 μm coronal sections through the hippocampal region. Free-floating sections were treated with 3% H_2O_2 in PBS for 10 min. Sections were blocked in 3% Normal Goat Serum (NGS) in PBS-X (0.3% Triton X-100), then incubated at 4 °C in primary antibody (1:1000) against synaptophysin (mouse IgG1 isotype; SVP-38, Sigma–Aldrich) in 3% NGS in PBS-X for 48 h. As a control for antibody specificity, a section was also incubated in the absence of synaptophysin; similarly, the primary antibody was omitted as a control in Western blotting (see below). In each case, omission of the primary antibody resulted in absence of visible immunoreactivity. Sections were incubated in a biotin-conjugated secondary antibody (horse, anti-mouse; 1:200) in 3% NGS in PBS-X for 1 h at room temperature. After incubation in an avidin–biotin complex in PBS-X for 1 h at room temperature, sections were developed with diaminobenzidine (DAB) (10 mg/100 ml 0.1 M PBS and 35 μl H_2O_2) for 35 s.

2.5.1. Optical densitometry 1

Optical densitometric (OD) analyses were done with Scion Image Software (Version 1.62), a light microscope (Olympus, BH-2) at 40× magnification, and a digital camera (COHU, High Performance CCD Camera) connected to a computer. Digitized images of brain sections were acquired with gray level intensities set as 0–255 (black to white). Measurements were obtained from three representative cross-sections of the hippocampus per animal, with care taken to match sections between animals. Similar to the method of Andersen and Teicher (2004), a square of ~2000 pixels was used to acquire two OD measurements from each of three hippocampal regions per section: dentate gyrus (DG), CA1 and CA3 in the left hemisphere. In the DG, two OD measurements were obtained from the molecular layer (DGm; primarily inner DGm). In both the CA1 and CA3, measurements were obtained from the oriens and radial strata (CA1o/r and CA3o/r, respectively). One measurement was obtained from the corpus callosum (CC) and the primary somatosensory cortex (Fig. 1). To account for illumination and immunostaining variability, OD measurements from the CC were subtracted from those obtained from each of the hippocampal regions and the cortex (corrected OD value).

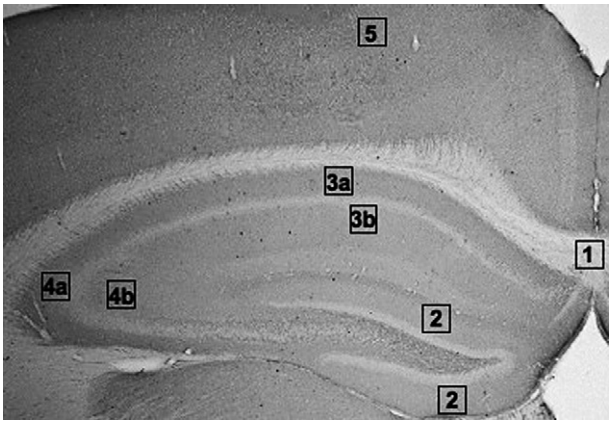

Fig. 1. Representative coronal section of gerbil hippocampus reacted with an antibody against synaptophysin and visualized with DAB. Boxes (~2000 pixels, 40×) are representative of regions where optical densitometry (OD) measurements were obtained. 1: Corpus callosum; 2: Dentate gyrus molecular layer (DGm); 3a: CA1 oriens (CA1o); 3b: CA1 radial layer (CA1r); 4a: CA3o; 4b: CA3r; 5: Cortex.

2.5.2. Optical densitometry 2

A second approach to OD measurement was used that differed in that measurements were made within a small circle containing ~400 pixels (40×) from each of the regions. Similarly to the method of Gao et al. (2006), eight measurements were taken from both the CA3o and the CA3 lucida and radiata (CA3 l/r) strata, immediately adjacent to the pyramidal layer. Eight measurements were also obtained from each of the CA1o and CA1r strata, immediately adjacent to the pyramidal layer. In the DG, 16 measurements were obtained from the inner molecular layer of the DG (DGm) and 16 were obtained from the hilus of the DG (DGh). Eight measurements were also obtained from the CC and the cortex. Measurements were obtained from one section per animal with care taken to match sections between animals (Fig. 2).

For both OD analyses, corrected OD averages and SEM were calculated for each of the hippocampal regions and the cortex, for each of the four groups. Using OD method 1, means were obtained by averaging measurements from the three sections examined in each region. Two-factor ANOVAs were used to test for effects of sex and postnatal condition on OD measurements from the examined regions for both methods; these were followed by Bonferroni *post hoc* tests.

2.6. Western immunoblotting

Animals used for Western blot analysis were killed with anesthetic overdose, brains were removed, immersed in cold isopentane and stored at −80 °C. Hippocampi were dissected out and then homogenized with ice-cold lysis buffer consisting of 150 mM NaCl, 50 nM Tris–HCl, 0.1% Triton X-100, 10% glycerol, 0.25% Na-deoxycholate, 1 mM EDTA and a cocktail of protease inhibitors (1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM phenylmethylsulfonyl fluoride; all Sigma–Aldrich). Following homogenization, samples were centrifuged (6000 rpm for 30 min at 4 °C) and the supernatant collected.

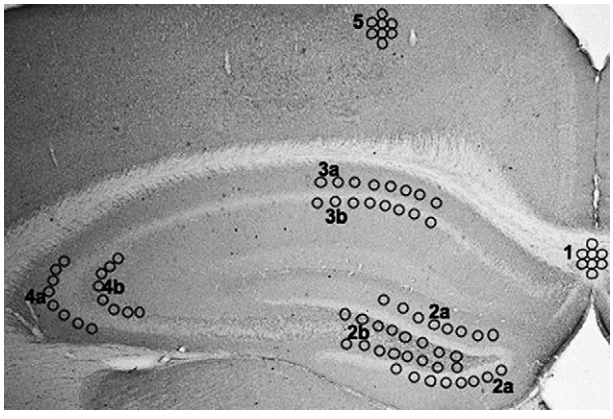

Fig. 2. Representative coronal section of gerbil hippocampus reacted with an antibody against synaptophysin and visualized with DAB. Open circles (~40 pixels, 40×) represent the regions from which optical densitometry (OD) measurements were obtained. 1: Corpus callosum; 2a: Dentate gyrus molecular layer (DGm); 2b DG hilus (DGh); 3a: CA1 oriens (CA1o); 3b: CA1 radial layer (CA1r); 4a: CA3o; 4b: CA3r; 5: Cortex.

Table 1
Body weights of postnatally separated (S) and control (C) gerbils on postnatal days (PND) 20, 56 and 80

Group	PND 20	PND 56	PND 80
S Males (n=29)	15.48±0.35 [a]	63.80±0.81	71.76±0.98
C Males (n=35)	16.56±0.41	66.16±1.34	72.88±0.98
S Females (n=43)	15.56±0.32	54.33±0.81	63.44±0.84 [a]
C Females (n=39)	16.33±0.30	52.96±0.75	60.41±0.92

[a] Weight (g) significantly different from C group ($p<0.05$). Values expressed as mean weight±SEM.

Samples (30 μg of protein as determined by the Bradford assay; Sigma–Aldrich) were denatured at 95 °C for 5 min. Protein was separated by electrophoresis on 15% polyacrylamide gels or precast gels (4–20% Tris-Glycine Gel; Invitrogen) at 225 V for 1 h. Protein was transferred to polyvinyl difluoride membranes at 250 mA for 2 h. Membranes were washed in 0.1 M Tris-buffered saline containing 0.1% Triton-X-100 (T-TBS) and blocked for 1 h at room temperature in 5% non-fat milk in T-TBS. Membranes were then incubated overnight (4 °C) with a rabbit polyclonal antibody to BDNF (1:500; Chemicon International, Inc., Temecula, CA, USA) in 1% non-fat milk in T-TBS. This was followed by incubation in the appropriate horseradish peroxidase (HRP)-linked secondary antibody (1:1000) in 1% non-fat milk in T-TBS for 30 min at room temperature.

Immunoreactive bands were visualized by chemiluminescence (LumiGlo solution; KPL Inc.) using a Kodak Imaging Station 400CF. Following blot visualization, membranes were stripped for 1 h at 70 °C in a stripping rinse (solution of 2% sodium dodecyl sulphate, 62.5 mM Tris–HCl, 100 mM β-mercaptoethanol in dH2O), washed twice (solution of 10 mM Tris–HCl, 150 mM NaCl in dH_2O) and blocked for 2.5 h in 5% non-fat milk in T-TBS. Membranes were reblotted with a mouse monoclonal antibody against synaptophysin (same as for IHC) and visualized. Membranes were stripped again, blocked and blotted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:20,000, Chemicon International, Inc.), incubated in the appropriate HRP-linked secondary antibody (1:5000) and visualized for the last time. Band intensity was analyzed using an analysis software package (KODAK 1D 3.6 Image Analysis Software). The ratios of band immunoreactivities corresponding to synaptophysin (38 kDa) and to three forms of BDNF (14 kDa: mature form; 27 kDa: BDNF dimer; 36 kDa: proform of BDNF) to GAPDH immunoreactivity (37 kDa) were calculated; these are referred to as corrected values. For each group, the mean of the corrected values and associated SEM values were calculated. Independent sample t tests were used to assess group differences for each sex as protein samples from only one sex were run per gel.

3. Results

3.1. Body weights

Pup weights (g) on PND 20 were evaluated by independent sample t tests for each sex. Among males, S pups weighed

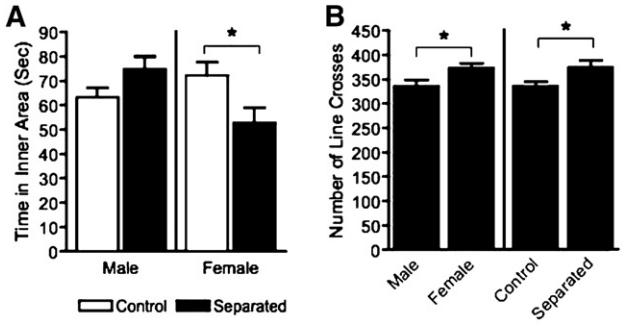

Fig. 3. Effect of postnatal separation on behavior during a 5 min open field test (OFT) in male and female adult gerbils. A. Time spent in inner area of the open field (mean±SEM, sec). *Significant difference between control and separated females, $p<0.05$. B. Number of lines crossed during the OFT (mean±SEM). *Significant main effect of sex (left) and postnatal treatment (right), $p<0.05$. Female n: S=11, C=8; Male n: S=9, C=8. All animals 80–90 days of age on day of testing.

significantly less than C pups [$t(1,62)=2.01$, $p<0.05$]. While there was no significant weight difference between the female groups, S pups tended to weigh less than C pups ($t(1,80)=1.76$, $p=0.082$; Table 1). No group differences were noted in developmental markers, such as fur development, eye opening and unfolding of the ears for either sex. On PND 56 no significant difference in body weight existed between S and C gerbils of either sex. By PND 80 however, S females weighed significantly more than C females [$t(1,80)=2.44$, $p<0.05$; Table 1].

3.2. Behavior in the open field test

Three of 11 C females were eliminated from this analysis because they had seizures in the open field (final $n=8$), while no S females exhibited seizures ($n=11$). Three of 11 C males, and 3 of 12 S males were eliminated from further analyses due to seizures in the open field (final $n=8$ and 9, respectively). There was no main effect of either sex or postnatal condition on time spent in the inner area of the open field but there was a significant interaction [$F(1,31)=8.75$, $p<0.01$]. The Bonferroni *post hoc* test indicated that S females spent significantly less time in the inner area of the open field than did C females ($p<0.05$; Fig. 3A). There was a main effect of sex ($F(1,31)=4.68$, $p<0.05$; Fig. 3B) and separation ($F(1,31)=5.22$, $p<0.05$; Fig. 3B) on number of line crosses, but no interaction. Although the Bonferroni *post hoc* test revealed no significant differences, there was a strong tendency for S females to cross more lines than C females ($p=0.061$). No other behavioral measures differed significantly over the entire test.

To assess whether postnatal condition affected initial or later responses in the open field, we analyzed behaviors separately for the first and last minutes of the 5 min test. There was no effect of postnatal condition and no interaction during the first minute for time spent in the inner area, but there was a significant main effect of sex [$F(1,31)=6.51$, $p<0.05$; Fig. 4A], with males spending more time in the inner area than females. There were no main effects for time spent in the inner area during the last minute, but there was a significant sex by postnatal treatment interaction [$F(1,31)=5.69$, $p<0.05$], reflecting the fact that S males spent significantly more time in the inner area than C males during the last minute ($p<0.05$; Fig. 4B). There were no significant differences in line crossing or rearing activity in these data.

Repeated measures ANOVAs were used to compare results from the first and last minutes of the test. Line crossing was reduced for both females and males in the last minute relative to the first minute, with no interaction with postnatal condition [females: $F(1,16)=12.00$, $p<0.01$; males ($F(1,15)=11.94$, $p<0.01$; Fig. 4B]. Females also showed a significant increase in average amount of rearing in the last minute [($F(1,16)=7.432$, $p<0.05$; 14.9 versus 18.9 rearing incidents in the first and last minutes, respectively], with no interaction with postnatal condition.

3.3. Behavior in the tail suspension test

No gerbils of either group or sex exhibited seizures during the TST; 11 C and 10 S males and 11 C and S females were used in the final analyses. There were no main effects of sex or postnatal treatment on duration of immobility during either the entire 6 min test or during the last 4 min of testing.

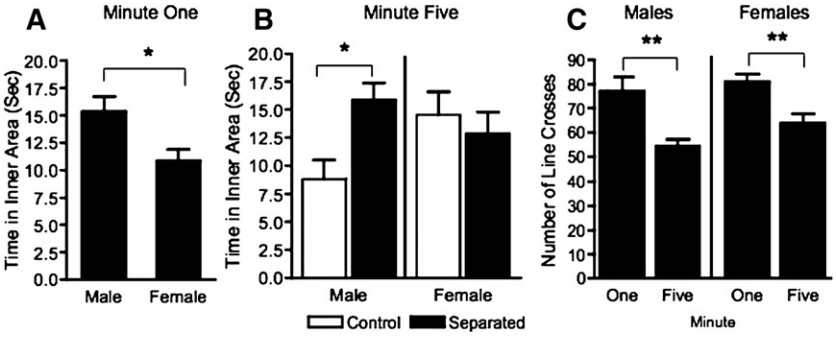

Fig. 4. Effect of postnatal separation on behavior in the first and last minutes of a 5 min open field test (OFT) in adult gerbils. A. Time spent in inner area of the open field by females and males during the first minute of testing (mean±SEM, s). *Significant difference between males and females, $p<0.05$. B. Time spent in inner area of the open field by females and males during the last minute of testing (mean±SEM, s).*Significant differences between control and separated animals, $p<0.05$. C. Number of lines crossed by females and males during the first and last minutes of testing (mean±SEM). **Significant differences between the first and last minutes in each sex, $p<0.01$. See legend for Fig. 3 for details.

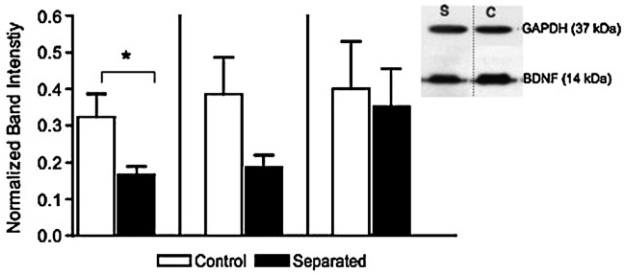

Fig. 5. Densitometric analysis of band intensities from Western blot analyses corresponding to the three forms of BDNF: mature form (14 kDa), dimer (28 kDa) and proform (36 kDa) in separated (S) and control (C) male gerbils. *Significantly decreased relative to C animals, $p<0.05$. Mean density ± SEM. S: $n=7–10$; C: $n=8$.

3.3.1. Optical densitometry 1

Two-factor ANOVAs revealed no significant effect of either sex or postnatal condition on synaptophysin immunoreactivity in the three hippocampal regions examined (DG, CA1 and CA3), nor in the cortex ($n=13$ S, 10 C females; $n=10$ S; 6 C males). There was a trend for a main effect of separation on OD measurements in the CA1 [$F(1,35)=3.46$, $p=0.071$]. The Bonferroni *post hoc* test revealed a trend toward a difference between C and S males ($p=0.071$), with S males tending to have less synaptophysin immunoreactivity in the CA1 region.

3.3.2. Optical densitometry 2

Using small circular templates aimed more specifically at hippocampal subfields did not alter these results, as no significant effect of sex or postnatal treatment was found.

3.4. BDNF and synaptophysin levels

Western blot studies were conducted in order to assess synaptophysin and BDNF levels in the entire hippocampus. There was a trend toward a decrease in hippocampal synaptophysin levels in S relative to C males, but this was not statistically significant [$t(1,17)=1.984$, $p=0.064$]. There was a significant group difference in hippocampal BDNF levels in the hippocampus, with S males showing lower values of the 14 kDa band representing BDNF than did C males [$t(1,13)=2.24$, $p<0.05$]. There were no significant differences in the mean intensity of the 28 kDa band (corresponding to BDNF dimers), although there was a tendency toward decreased immunoreactivity in S males [$t(1, 14)=1.90$, $p=0.079$], nor of the 36 kDa band (dominant pro-form of BDNF; Fig. 5).

Although the means for all three BDNF bands were lower for S than for C females, there were no statistically significant differences between groups for either hippocampal BDNF or synaptophysin (C, $n=7–8$; S, $n=9$).

4. Discussion

Our results indicate that separation induces subtle behavioral and neurochemical changes in gerbils that manifest differently in the two sexes.

4.1. Body weight

S males weighed less than C males on PND 20, and S females exhibited the same tendency, but had overtaken their controls by PND 80. A weight decrease in separated pups has been observed in studies of other species (Ruedi-Bettschen et al., 2004), and is probably related in part to lost nursing time. Other physiological effects of separation, however, may also play a role (Kuhn and Schanberg, 1998), and may contribute to the subsequent increase in body weight of S females as adults.

4.2. Behavioral studies

Consistent with studies in other rodent species, we observed that females crossed more lines in the open field than males, a result that has been attributed to gonadal hormone differences (Masur et al., 1980; Slob et al., 1981). S females also spent significantly less time than controls in the inner region of the open field. Since treatment with anxiolytics and some antidepressants increases the time rodents spend in the inner region in such tests (Borsini et al., 2002; Prut and Belzung, 2003), this observation suggests that avoidance of this area reflects an anxiety-like state or increased stress in the novel environment (Gregus et al., 2005; Ramos and Mormede, 1998).

In contrast to the females, S males did not differ from controls on behavioral measures during the 5 min OFT. This finding contrasts with some reports that male rodents exhibit more anxious-like behavior than females after postnatal separation (Boccia and Pedersen, 2001; Kalinichev et al., 2002; Wigger and Neumann, 1999), although a recent study found more pronounced effects of separation on the HPA axis, body weight and locomotor activity of female rats relative to males (Slotten et al., 2006). These differences emphasize the importance of including both sexes in studies of models of mood disorders.

Activity during initial exposure to the open field (first 30–60 s) is considered to be informative about the initial degree of fear, and stress experienced (Duncko et al., 2001; Kalynchuk and Meaney, 2003; Westenbroek et al., 2003), while changes in activity by the final minute of a test reflect the process of adaptation to the novel environment. Consistent with the process of adaptation leading to reduced exploration, gerbils of both sexes decreased line crosses from the first to last minute of the test. Females in both groups, however, increased rearing activity in the last minute. Although rearing is also a form of exploration, horizontal locomotor activity and rearing have been suggested to be mediated by different brain mechanisms (Dandiya and Kulkarni, 1974), and rearing may be more probable in a later stage of exploration.

It is surprising that S males spent significantly more time in the inner area than controls during the last minute, since groups did not differ overall or in the first minute. This result appears to reflect a decrease in time spent in the inner area during that minute by C males, which was not shown by any of the other three groups. There was a weak tendency for C males to spend more time grooming, rearing and jumping during the last minute, all of which occurred almost exclusively in the periphery of the open field (C males: mean = 30.00 s ± 1.73 SEM versus S males: mean = 26.49 s ± 1.15 SEM; t(1,15) = 1.73,

$p=0.1$). A focus on activities near the wall that might be escape-motivated (rearing, jumping) or a displacement activity (grooming) may have contributed to C males spending less time in the inner area during the last minute.

Since all females were studied during the estrous phase of their cycles, behavioral differences between groups in the OFT may reflect either a general difference between S and C females or differential responsiveness to the still-elevated levels of estrogen present at this stage. One study reported that S female rats exhibited reduced anxiety in the OFT during diestrus, but behaved similarly to controls during estrus (Romeo et al., 2003). Although the results in gerbils and rats differ, the fact that separated rats became more anxious during estrus relative to diestrus is consistent with our findings in gerbils. A more complete analysis of the interaction between estrous phase/hormonal status in adulthood and neonatal maternal separation is needed in gerbils.

The effects of early separation on behaviors considered to be ethologically relevant markers of stress in gerbils, such as foot-thumping, stereotyped digging and seizures (Laming et al., 1989; Ludvig et al., 1991; Woolley et al., 2006) could not be assessed in this study. Foot-thumping and digging occurred at too low a frequency to permit meaningful analysis. Gerbils that exhibited seizures almost invariably became immobile for the remainder of the test period, so they had to be excluded from the analysis.

The tail suspension test (TST) was used in this study because it has been validated previously in gerbils as a measure of learned helplessness (Varty et al., 2003), although it has not been used previously in a neonatal separation paradigm. The lack of any treatment effect in either sex on the TST in this study may be related to the fact that it both assesses and induces learned helplessness; repeated TST sessions might reveal an effect of separation. The well-studied forced swim test (FST; Porsolt et al., 1977) could not be used because pilot studies indicated that gerbils are poor swimmers, and their tendency to show seizures under stress made the test dangerous for them. Similarly, the social interaction test could not be used because of the prevalence of seizures and aggression in pilot studies.

4.3. Neurochemical studies

We found a significant decrease in the mature (14 kDa) form of BDNF in the hippocampus of neonatally separated male gerbils relative to controls, and a trend toward a similar difference in the 28 kDa BDNF dimer. One study in male rats reported a significant reduction in BDNF mRNA in prefrontal cortex (but not hippocampus) and of BDNF protein in hippocampus after neonatal separation (Roceri et al., 2004), but another reported a significant increase in BDNF protein in S male rats (Greisen et al., 2005). While our results in male gerbils are generally consistent with those of Roceri et al. (2004), there are too few studies, especially comparing across species, to draw any strong conclusions.

One previous study, in which there was no sex difference observed, collapsed data across sex and found a difference in the expression of synaptophysin between separated and control adult rats (Andersen and Teicher, 2004). Another study (Bisagno et al., 2004), however, did not find differences in synaptophysin levels between control and stressed females (although estrous phase was not accounted for in that study). While we did not observe significant differences in synaptophysin levels in either sex, there was a trend toward a decrease in synaptophysin expression in S males relative to controls in the CA1 region using one densitometric method, which is consistent with previous results in rats using that method (Andersen and Teicher, 2004). There was also a tendency for decreased synaptophysin levels assessed using Western blots in S males. It remains unclear whether hormonal status affects levels of hippocampal synaptophysin and how such effects might interact with a history of neonatal stress. The trends observed, however, are consistent with the decreased BDNF levels and a possible reduction in synapse number in S males, since BDNF plays a critical role in neuronal survival and maintenance of synaptic integrity.

The neurochemical changes observed in the hippocampus of male gerbils may be expected to be related to behavioral differences. Female gerbils, however, showed the principal behavioral changes related to separation but no significant neurochemical changes; so there is no obvious linkage between the neurochemical and behavioral changes observed. Future studies will need to explore linkages between neurochemical changes induced by neonatal separation and other behavioral assays once these have been validated in gerbils.

4.4. Limitations and conclusions

One limitation of this study is that separation started on PND 4 and earlier separation might have induced more robust effects. In the absence of previous separation studies on gerbils, we were concerned that earlier separation might have been too stressful. The lack of pup mortality in this study, however, suggests that earlier separation may be feasible in future studies. Another limitation is that the daily handling (although similar in the two treatment groups) may have attenuated potential group differences in anxiety-like behaviors. This handling was necessary because of the tendency for unhandled adult gerbils to have seizures in response to simply being brought into the testing room, but methods that do not require such extensive handling would be preferable.

Despite these limitations, the results reinforce the conclusion that male and female rodents respond differently to early neonatal separation. The results suggest that the neurochemical targets studied did not relate to the behavioral effects observed. Given that the motivation to study gerbils is related in part to the characteristics of their neurokinin receptors, studies aimed specifically at parts of the SP system might be of interest. Additional research comparing males and females, assessing the role of hormonal status in modulating developmental effects on behavior and neurochemistry, and targeting other neural mechanisms in gerbils are clearly required.

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References

Altieri M, Marini F, Arban R, Vitulli G, Jansson BO. Expression analysis of brain-derived neurotrophic factor (BDNF) mRNA isoforms after chronic and acute antidepressant treatment. Brain Res 2004;1000:148–55.

Andersen SL, Teicher MH. Delayed effects of early stress on hippocampal development. Neuropsychopharmacology 2004;29:1988–93.

Benninghoff J, Schmitt A, Mossner R, Lesch KP. When cells become depressed: focus on neural stem cells in novel treatment strategies against depression. J Neural Transm 2002;109:947–62.

Bisagno V, Grillo CA, Piroli GG, Giraldo P, McEwen B, Luine VN. Chronic stress alters amphetamine effects on behavior and synaptophysin levels in female rats. Pharmacol Biochem Behav 2004;78:541–50.

Boccia ML, Pedersen CA. Brief vs. long maternal separations in infancy: contrasting relationships with adult maternal behavior and lactation levels of aggression and anxiety. Psychoneuroendocrinology 2001;26:657–72.

Borsini F, Podhorna J, Marazziti D. Do animal models of anxiety predict anxiolytic-like effects of antidepressants? Psychopharmacology (Berl) 2002;163:121–41.

Conrad CD, LeDoux JE, Magarinos AM, McEwen BS. Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. Behav Neurosci 1999;113:902–1000.

Coyle JT, Duman RS. Finding the intracellular signaling pathways affected by mood disorder treatments. Neuron 2003;38:157–60.

Czeh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, et al. Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proc Natl Acad Sci U S A 2001;98:12796–801.

Dandiya PC, Kulkarni SK. A comparative study of d- and l-amphetamine on the open field performance of rats. Psychopharmacologia 1974;39:67–70.

Duman RS, Monteggia LM. A neurotrophic model for stress-related mood disorders. Biol Psychiatry 2006;59:1116–27.

Duncko R, Kiss A, Skultetyova I, Rusnak M, Jezova D. Corticotropin-releasing hormone mRNA levels in response to chronic mild stress rise in male but not in female rats while tyrosine hydroxylase mRNA levels decrease in both sexes. Psychoneuroendocrinology 2001;26:77–89.

Fossati P, Radtchenkoa A, Boyera P. Neuroplasticity: from MRI to depressive symptoms. Eur Neuropsychopharmacol 2004;14(Suppl 5):503–10.

Gamaro GD, Streck EL, Matte C, Prediger ME, Wyse AT, Dalmaz C. Reduction of hippocampal Na+, K+-ATPase activity in rats subjected to an experimental model of depression. Neurochem Res 2003;28:1339–44.

Gao Y, Bezchlibnyk YB, Sun X, Wang JF, McEwen BD, Young LT. Effects of restraint stress on the expression of proteins involved in synaptic vesicle exocytosis in the hippocampus. Neuroscience 2006;141:1139–48.

Gilmer WS, McKinney WT. Early experience and depressive disorders: human and non-human primate studies. J Affect Disord 2003;75:97–113.

Gitter BD, Waters DC, Bruns RF, Mason NR, Nixon JA, Howbert JJ. Species differences in affinities of non-peptide antagonists for substance P receptors. Eur J Pharmacol 1991;197:237–8.

Gregus A, Wintink AJ, Davis AC, Kalynchuk LE. Effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats. Behav Brain Res 2005;156:105–14.

Greisen MH, Altar CA, Bolwig TG, Whitehead R, Wortwein G. Increased adult hippocampal brain-derived neurotrophic factor and normal levels of neurogenesis in maternal separation rats. J Neurosci Res 2005;79:772–8.

Hajszan T, MacLusky NJ, Leranth C. Short-term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. Eur J Neurosci 2005;21:1299–303.

Heim C, Plotsky PM, Nemeroff CB. Importance of studying the contributions of early adverse experience to neurobiological findings in depression. Neuropsychopharmacology 2004;29:641–8.

Holden C. Future brightening for depression treatments. Science 2003;302:810–3.

Huot RL, Thrivikraman KV, Meaney MJ, Plotsky PM. Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. Pychopharmacology (Berl) 2001;158:366–73.

Kalynchuk LE, Meaney MJ. Amygdala kindling increases fear responses and decreases glucocorticoid receptor mRNA expression in hippocampal regions. Prog Neuro-Psychopharmacol Biol Psychiatry 2003;27:1225–34.

Kalinichev M, Easterling KW, Plotsky PM, Holtzman SG. 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 2002;73:131–40.

Keller M, Montgomery S, Ball W, Morrison M, Snavely D, Liu G, et al. Lack of efficacy of the substance P (neurokinin1 receptor) antagonist aprepitant in the treatment of major depressive disorder. Biol Psychiatry 2006;59:216–23.

Kramer MS, Cutler N, Feighner J, Shrivastava R, Carman J, Sramek JJ, et al. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. Science 1998;281:1640–5.

Kuhn CM, Schanberg SM. Responses to maternal separation: mechanisms and mediators. Int J Dev Neurosci 1998;16:261–70.

Laming PR, Elwood RW, Best PM. Epileptic tendencies in relation to behavioral responses to a novel environment in the Mongolian gerbil. Behav Neural Biol 1989;51:92–101.

Ludvig N, Farias PA, Ribak CE. An analysis of various environmental and specific sensory stimuli on the seizure activity of the Mongolian gerbil. Epilepsy Res 1991;8:30–5.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 2000;20:9104–10.

Magariños AM, McEwen BS. Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons comparison of stressors. Neuroscience 1995;69:83–8.

Masur J, Schutz MT, Boerngen R. Gender differences in open-field behavior as a function of age. Dev Psychobiol 1980;13:107–10.

Nishino N, Totsukawa K. Study of the estrous cycle in the Mongolian gerbil (*Meriones unguiculatus*). Exp Anim 1996;45:283–8.

O'Connor TG, Cameron JL. Translating research findings on early experience to prevention:animal and human evidence on early attachment relationships. Am J Prev Med 2006;31(6 Suppl 1):175–81.

Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature 1977;266:730–2.

Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003;463:3–33.

Pryce CR, Bettschen D, Bahr NI, Feldon J. Comparison of the effects of infant handling, isolation and non-handling on acoustic startle, prepulse inhibition, locomotion, and HPA activity in the adult rat. Behav Neurosci 2001;115:71–83.

Pryce CR, Bettschen D, Nanz-Bahr NI, Feldon J. Comparison of the effects of early handling and early deprivation on conditioned stimulus, context, and spatial learning and memory in adult rats. Behav Neurosci 2003;117:883–93.

Rajowska G. Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. Biol Psychiatry 2000;48:766–77.

Ramos A, Mormede P. Stress and emotionality: a multidimensional and genetic approach. Neurosci Biobehav Rev 1998;22:33–57.

Roceri M, Hendriks W, Racagni G, Ellenbroek BA, Riva MA. Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. Mol Psychiatry 2002;7:609–16.

Roceri M, Cirulli F, Pessina C, Peretto P, Racagni G, Riva MA. Postnatal repeated maternal deprivation produces age-dependent changes of brain-derived neurotrophic factor expression in selected rat brain regions. Biol Psychiatry 2004;55:708–14.

Romeo RD, Mueller A, Sisti HM, Ogawa S, McEwen BS, Brake WG. Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. Horm Behav 2003;43:561–7.

Ruedi-Bettschen D, Feldon J, Pryce CR. Circadian- and temperature-specific effects of early deprivation on rat maternal care and pup development: short-term markers for long-term effects? Dev Psychobiol 2004;45:59–71.

Rupniak NMJ, Carlson EC, Harrison T, Oates B, Seward E, Owen S, et al. Pharmacological blockade or genetic deletion of substance P (NK1) receptors attenuates neonatal vocalisation in guinea-pigs and mice. Neuropharmacology 2000;39:1413–21.

Russo-Neustadt AA, Chen MJ. Brain-derived neurotrophic factor and antidepressant activity. Curr Pharm Des 2005;11:1495–510.

Salome N, Stemmelin J, Cohen C, Griebel G. Selective blockade of NK2 or NK3 receptors produces anxiolytic- and antidepressant-like effects in gerbils. Pharmacol Biochem Behav 2006;83:533–9.

Santarelli L, Gobbi G, Debs PC, Sibille ET, Blier P, Hen R, et al. Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. Proc Natl Acad Sci U S A 2001;98:1912–7.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, et al. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 2003;301:805–9.

Slob AK, Bogers H, van Stolk MA. Effects of gonadectomy and exogenous gonadal steroids on sex differences in open field behaviour of adult rats. Behav Brain Res 1981;2:347–62.

Slotten HA, Kalinichev M, Hagan JJ, Marsden CA, Fone KC. Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: Gender-dependent effects. Brain Res 2006;1097:123–32.

Tartaglia N, Du J, Tyler WJ, Neale E, Pozzo-Miller L, Lu B. Protein synthesis-dependent and -independent regulation of hippocampal synapses by brain-derived neurotrophic factor. J Biol Chem 2001;276:37585–93.

Varty GB, Cohen-Williams ME, Hunter JC. The antidepressant-like effects of neurokinin NK1 receptor antagonists in a gerbil tail suspension test. Behav Pharmacol 2003;14:87–95.

Westenbroek C, Ter Horst GJ, Roos MH, Kuipers SD, Trentani A, den Boer JA. Gender-specific effects of social housing in rats after chronic mild stress exposure. Prog Neuro-Psychopharmacol Biol Psychiatry 2003;27:21–30.

Wigger A, Neumann ID. Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav 1999;66:293–302.

Woolley ML, Haman M, Higgins GA, Ballard TM. Investigating the effect of bilateral amygdala lesions on fear conditioning and social interaction in the male Mongolian gerbil. Brain Res 2006;1078:151–8.

Zimmerberg B, Rosenthal AJ, Stark AC. Neonatal social isolation alters both maternal and pup behaviors in rats. Dev Psychobiol 2003;42:52–63.