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The Effects of Early Rearing Environment on the Development of $GABA_A$ and Central Benzodiazepine Receptor Levels and Novelty-Induced Fearfulness in the Rat

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We compared the effects of handling or maternal separation from the day following birth until postnatal day 14 on behavioral responses to novelty and on $GABA_A$ and central benzodiazepine (CBZ) receptor levels in the rat. As adults, handled animals showed reduced startle responsivity, increased exploration in a novel open field, and decreased novelty-induced suppression of feeding relative to the handled (H) and/or maternal separation (MS) groups. As compared with handled animals, both nonhandled (NH) and MS animals displayed: (1) reduced $GABA_A$ receptor levels in the locus coeruleus (LC) and the n. tractus solitarius (NTS); (2) reduced CBZ receptor sites in the central and lateral n. of the amygdala, the frontal cortex, and in the LC and NTS; and (3) reduced levels of the mRNA for the $\gamma 2$ subunit of the $GABA_A$ receptor complex, which confers high affinity BZ binding, in the amygdaloid nuclei as well as in the LC and NTS. Both the amygdala and the ascending noradrenergic systems have been considered as critical sites for the anxiolytic effects of benzodiazepines. These data suggest that early life events influence the development of the $GABA_A$ receptor system, thus altering the expression of fearfulness in adulthood.
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KEY WORDS: *Fear; Novelty; $GABA_A$ receptor; Central benzodiazepine receptor; Maternal separation; Early experience*

The development of behavioral and endocrine responses to acute stress is greatly influenced by the early postnatal rearing environment (for reviews see Levine 1975; Denenberg 1964; Meaney et al. 1996). These environmental effects persist throughout the life of the animal, resulting in stable individual differences in stress reactivity. Indeed, there is considerable plasticity in the development of these systems. Postnatal handling during the first week of life greatly decreases behavioral fearfulness and hypothalamic-pituitary-adrenal (HPA) responses to stress; whereas, repeated periods of prolonged maternal separation produce enhanced reactivity. Increased stress reactivity has been associated with an enhanced risk for several forms of illness, including affective disorders, diabetes, autoimmune disorders, and coronary heart disease (Chrousos and Gold 1992; Higley et al. 1991; McEwen and Steller 1993; Seckl and Meaney 1994). Thus, in determining the magnitude of behavioral and endocrine responses to stress, early life events

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contribute to vulnerability to disease in later life (Seckl and Meaney 1994). The critical question, then, concerns the mechanisms for these early environmental effects on the development of behavioral and endocrine responses to stress.

Postnatal handling has been shown to decrease fearfulness to novelty (Levine 1962, 1957; Denenberg 1964; Bodnoff et al. 1987). As adults, handled (H) rats show reduced novelty-induced suppression of appetitive behavior and increased exploration in novel environments. The behavioral effects of repeated maternal separation in rats are less documented, although, in primates, there is considerable evidence for enhanced fearfulness in animals exposed to maternal separation in early life (Sackett 1969).

The mechanisms underlying these early environmental effects on behavioral responses to novelty are unclear. Bodnoff et al. (1987) reported that neonatal handling increased forebrain central benzodiazepine (CBZ) receptor levels, with no indication of where in the forebrain such differences might exist. Nevertheless, these data are certainly consistent with the well-established, anxiolytic effects of benzodiazepines on behavioral responses to novelty (see File 1995 for a review). Moreover, these findings are also consistent with human data showing decreased CBZ receptor sensitivity in panic disorder patients (Roy-Byrne et al. 1996).

We report here the results of behavioral and pharmacological studies examining various behavioral responses to novelty as well as differences in CBZ and $GABA_A$ receptor binding levels in adult animals exposed to either handling or maternal separation during the first 2 weeks of life. The results of these studies are consistent with the idea that the early environment can regulate the development of $GABA_A$/CBZ receptor systems and that these effects, in turn, partially mediate the differences in behavioral responses to stress.

MATERIALS AND METHODS

Subjects

The animals used in these studies were the male offspring of Long–Evans, hooded rat dams obtained from Charles River (St. Constant, Quebec or Boston, MA) and mated in our animal colony. Handling consisted of removing the mother and then the pups from the home cage by gloved hand and placing the pups into a plastic container lined with bedding material for 15 minutes. The pups, followed by the mother, were then returned to their home cage. Handling occurred once per day between postnatal days (PND) 1 (Day 0 = day of birth) and 14 of life. Maternal separation involved the same procedures; however, the pups remained away from the dam for 180 minutes. In the course of normal mother–pup interactions in the laboratory rat, the mother is routinely off the litter for periods of 20–25 minutes (the internest bout interval, see Leon et al. 1978; Jans and Woodside 1990). Thus, the maternal separation paradigm, unlike handling, represents the deprivation of maternal care. The nonhandled (NH) animals were left completely undisturbed throughout this period. For all animals, routine cage maintenance did not begin before PND 14.

On PND 22, the animals were weaned and housed in same-sex, same-treatment groups of three animals per cage. The animals were maintained on a 12:12 light:dark schedule (lights on at 0800 h) with free access to food (Purina Lab Chow) and water. The animals used in these experiments were 3 to 4 months of age (300–350 gm) at the time of testing and were randomly selected from approximately five litters per treatment group. There were no group differences in body weights. The experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the McGill University Animal Care Committee and in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Behavioral Testing

For all behavioral testing, observers were blind as to the treatment condition of the animal. Each behavioral experiment was performed with a separate set of animals that were not exposed to any previous testing.

Animals were tested one at a time using a modified version of the Britton and Thatcher-Britton paradigm as a measure of novelty-induced suppression of appetitive behavior (see Britton and Thatcher-Britton 1981; Bodnoff et al. 1987,1989). Animals were food deprived for 24 hours before testing. The animals were then provided with food either in a novel environment or in the home cage. The novel environment was a 180 × 180 × 30 cm arena with food provided in a cylindrical wire-mesh hopper located in the center of the novel arena. To begin the test, all animals were placed one at a time into the periphery of the arena at the same starting point. The test session lasted for 360 seconds, and during this period, the experimenter scored the latency (s) to begin feeding, the total amount of time spent feeding, and the latency to first "visit" the food (i.e., to move within a 5-cm radius of the food container). If an animal did not eat within the test period, a score of 360 was assigned for latency measures and 0 for the amount of food consumed. The novel arena was cleaned with ethanol following the testing of each animal. A separate group of animals were tested in the same manner, with the exception that food was provided in the home cage, using the same food hopper, rather than in the novel environment. Although normally housed in groups, for the

sake of testing, animals were tested alone in the home cage. The partner was removed 1 hour before testing.

Another set of animals was examined in an open-field test of exploration. Individual animals were placed, one at a time, into the same location of the periphery of a novel circular open field, 1.6 m in diameter. The critical measure was the time (s) the animal spent exploring the inner area of the novel arena. Exploration was defined as the entire body of the animal being away from the immediate vicinity of the wall (>10 cm) enclosing the open field. Each rat was tested for 10 min in the novel environment. The open field was cleaned between each subject to prevent olfactory cues from affecting the behavior of subsequently tested rats.

Finally, a third set of animals was tested for startle responses using two startle chambers (San Diego Instruments, San Diego, CA), each consisting of a *Plexiglas* chamber mounted on a *Plexiglas* base within a sound-attenuating chamber. A piezoelectric strain meter attached to the base transduced the startle response. Stabilimeter readings were rectified, digitized on a 4095 scale, and recorded by computer. An average of 100 1-ms readings, beginning at stimulus onset, was used as the measure of startle amplitude for each trial. A test session consisted of placing an animal in the startle chamber for a 5-min acclimatization period, after which it was exposed to five presentations of an 80 to 120 dB acoustic startle stimulus (30 ms) in a random sequence, separated by variable interstimulus intervals that averaged 15 s. The startle chambers were cleaned with a 70% alcohol solution between animals.

In Vitro $GABA_A$/Central Benzodiazepine Receptor Autoradiography

Handled, nonhandled, and maternal separation animals ($n = 6$/group) were rapidly decapitated less than 1 minute following removal from the home cage in order to avoid the dynamic effects of acute stress on BZ receptor expression (see Medina et al. 1983). Brains were quickly removed, frozen in −70°C isopentane, and stored at −80°C. Brains were sectioned at 15 μm, and sections were thaw-mounted onto gel-coated slides. Slides were then stored at −80°C until the time of assay.

Central benzodiazepine (CBZ) receptors were first examined using a procedure described by Bureau and Olsen (1993) with unfixed, frozen sections. Slides were thawed and pre-incubated in assay buffer (0.17 m Tris-HCl, pH 7.4) for 30 min at 4°C. The slides were then incubated with a saturating 0.5 nm concentration of [^{3}H]flunitrazepam (84.5Ci/mmol, New England Nuclear, Boston, MA) in assay buffer for 60 min at 4°C. Nonspecific, background binding was determined in parallel sections using 1 μm clonazepam. Postassay washes (2 × 30 s) were performed using assay buffer. The sections were left to dry overnight and then were apposed to tritium-sensitive Ultrafilm (Amersham Canada Inc., Montreal, Canada) along with ^{3}H microscales for 14 days.

Type I CBZ receptor binding was quantified using [^{3}H]zolpidem (50.8 Ci/mmol, New England Nuclear), following procedures described in Ruano et al. (1993). Slides were thawed and pre-incubated in 50 mm Tris-HCl assay buffer (120 mm NaCl, 5 mm KCl, pH 7.4) for 30 min at room temperature. Sections were then incubated in assay buffer containing a saturating 5 nm concentration of [^{3}H]zolpidem (5 nm) for 30 min at 4°C. Nonspecific binding was determined in parallel sections incubated with buffer containing 50 μm of cold zolpidem (purchased from RBI, Natick, MA). Sections were then washed (2 × 3 min) in ice-cold assay buffer, rinsed in cold, distilled water, and dried rapidly under a stream of cold air. Autoradiograms were generated by apposing slides to tritium-sensitive ultrafilm with the appropriate [^{3}H] microscales for a period of 14 days, a period that provided for linearity of microscales and maximal binding.

$GABA_A$ receptor levels were quantified using [^{3}H]GABA according to the procedure of Chu et al. (1990). After thawing, slides were pre-incubated in 50 mm Tris-HCl buffer (pH 7.4) for 60 min at room temperature. Slides were then incubated with 30 nm [^{3}H]GABA and 10 mm baclofen, to block binding to $GABA_B$ receptor sites, at 4°C for 45 min in 50 mm Tris-HCl 50 mm buffer (pH 7.4) containing 2.5 mm CaCl . Nonspecific background binding was determined on parallel sections by the addition of 10 mm isovagine. Following incubation, slides were washed (2 × 5 min) in ice cold 50 mm Tris-HCl buffer. The slides were then dipped quickly in acetone:glutaraldehyde 2.5% and dried under a gentle stream of air. Autoradiograms were generated by exposure to ^{3}H-sensitive ultrafilm along with ^{3}H microscale standards for 21 days.

Autoradiograms were analyzed by obtaining optical densities (expressed as mean ± SEM in fmol/mg protein) determined by computer-assisted densitometry using an MCID image analysis system (Imaging Research Inc., St. Catherine's, Ontario, Canada) and low activity tritium standards. For each brain region, nonspecific binding was assessed using analysis of the same brain region from sections prepared as described above. Sections were coded, and the analysis was performed by individuals unaware of the treatment origin of the sections.

In Situ Hybridization

Brains were obtained as described above, and 15 μm coronal sections were prepared under RNase-free conditions and stored at −80°C. In preparation for the hybridization experiments, sections were prefixed in a 4% paraformaldehyde solution for 10 min. Sections were then washed in 2 × SSC buffer (2 × 5 min) and in 0.25%

acetic anhydride and 0.1 m triethanolamine solution (pH 8.0; 1 × 10 min). Sections were then dehydrated using a 50 to 100% ethanol gradient, placed in choloroform for 10 min, followed by a rehydration in 95% ethanol. Sections were then incubated overnight at 37°C with 75 μl/section of hybridization buffer containing 50% deionized formamide, 10 mm dithiothreitol, 10 mm Tris (Ph 7.5), 600 mm sodium chloride, 1 mm EDTA, 10% dextran sulfate, 1 × Denhardt's solution, 100 μg salmon sperm DNA, 100 μg/ml yeast tRNA, with 1×10^6 CPM [^{35}S] ddATP labeled γ_2 oligonucleotide probe. The γ_2 oligonucleotide probe (5′–3′: GTC ATA GCC ATA TTC TTC ATC CCT CTC TTG AAG GTG GGT GGC; Shivers et al. 1989) was synthesized (Beckman 1000 DNA Synthesizer, Beckman, USA) and labeled using a DNA 3′-end labeling kit (Boehringer Mannheim, USA). Preliminary studies using a scrambled version of the probe yielded no specific signal on brain sections (data not shown). The slides were then dipped in NTB-2 emulsion (Kodak, Rochester, NY), stored at 4°C for 8 weeks, and developed. Following counterstaining with Cresyl Violet, the results were analyzed using grain counting over individual cells within high-power microscopic fields under brightfield illumination with an MCID image analysis system (Imaging Research Inc., St. Catherines, Ontario). For each individual cellfield, grains over every identifiable cell were counted, with 40 to 60 cells per area per section, with three sections per animal. Brain regions were identified using the rat brain atlas of Paxinos and Watson (1986). For the sake of the hippocampus, we focused on the pyramidal cell layers (CA1 and CA3) and the granule cell layers of the dentate gyrus. After subtraction of background (grains/neuropil), mean values were obtained for each animal. Background was less than 10% for all brain regions. Grain counts are presented as a function of the cell area

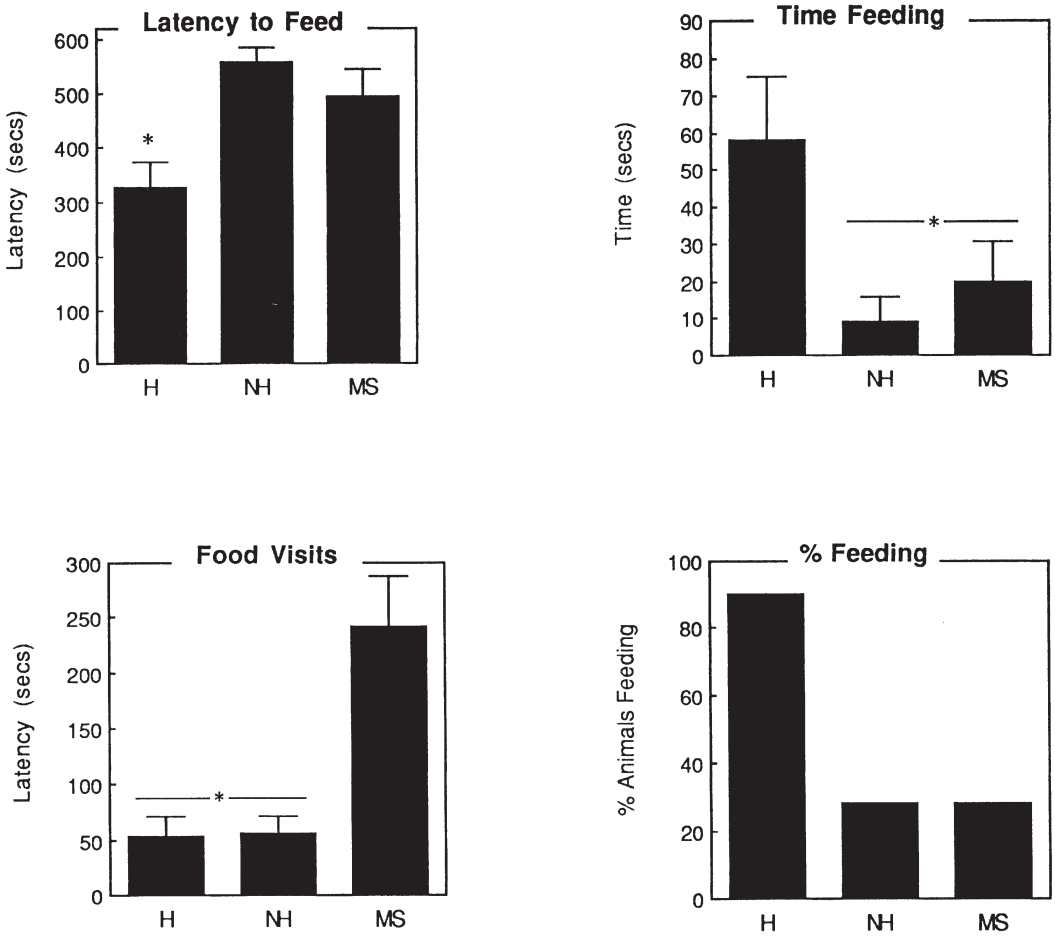

Figure 1. The results of the neophagia test for handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 7–10 per group). The mean ± SEM for the latency to begin feeding (upper left), the amount of time spent feeding (upper right), the latency to first visit to the food (lower left), and the percentage of animals within each group feeding at anytime during the test. *$p < .01$ from other groups; in cases where groups fall under a common line, the asterisks indicate a significant difference from the remaining group.

to account for any morphological differences (see McCabe et al. 1989).

Statistical Analysis

All data were analyzed using a one-way (group) or two-way (group × sample) analysis of variance (ANOVA) with Tukey post hoc tests, where appropriate.

RESULTS

Behavioral Testing

The results of the behavioral testing revealed consistent evidence for decreased fearfulness in the presence of novelty in adult animals exposed to handling during the first weeks of life, as compared with either NH or MS animals. In the neophagia test, food-deprived, handled (H) animals provided food in a novel setting ate significantly faster (i.e., a significantly shorter latency to feed, see Figure 1) and for a longer period of time (i.e., time eating, Figure 1) than did either NH or MS rats. NH and MS animals did not differ on these measures; however, the MS rats took significantly longer then either H or NH animals to approach the food source first (food visit latency; see Figure 1) than did either the H or the NH rats (see Figure 1). Overall, the percentage of either NH or MS rats that actually fed in the novel setting (see % feeding, Figure 1) was considerably lower in the NH and MS groups as compared with the H group. Note, there were no differences among the groups on any of these measures when food was provided animals in the home cage. Under these conditions, animals ate readily throughout the test period (the means for all groups were < 10 s; data not shown). The differences in the latency to begin feeding were apparent only in the novel environment, suggesting that the relevant variable was the animals' reaction to novelty, and not differences in hunger. To examine the responses to novelty further, animals were evaluated in an open-field test. In this test, H animals spent significantly more time exploring the center area (see Figure 2A), a measure that is thought to reflect the reduced fear in these animals. There were no differences between NH and MS rats.

Differences in fearfulness as a function of early rearing environment were also apparent in the results of the startle response test. H animals showed reduced startle responses, as compared with both NH and MS rats (see Figure 2B). At the higher decibel tones, there was also evidence for an increased startle response in the MS rats, as compared with the NH animals.

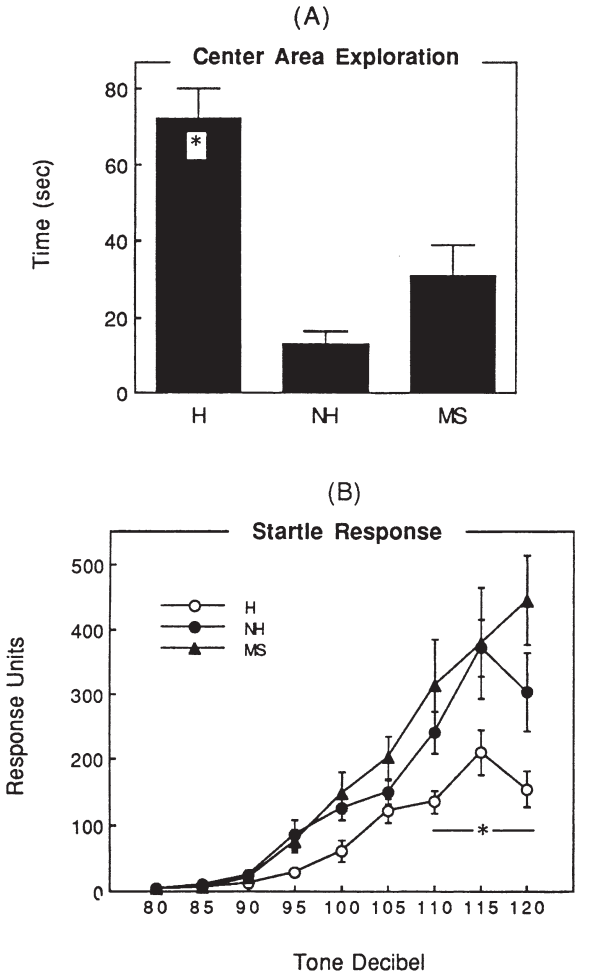

Figure 2. **(A)**The amount of time (mean ± SEM) exploring the center area of a novel open field arena in handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 8–10 per group, * $p < .01$ from other groups). **(B)** The startle response (mean ± SEM) in handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 8–11 per group) in response to 80–120 decibel tones (* indicates significant difference at $p < .05$ between H and NH or MS rats for each datapoint indicated by the solid line).

$GABA_A$/CBZ Receptor Levels

There were no differences in [^{3}H]GABA binding ($GABA_A$ receptor levels) in any corticolimbic area examined in this study, except for the medial prefrontal cortex region (see Figure 3A). In the medial prefrontal cortex, [^{3}H]GABA binding was significantly higher in H as compared to MS rats. In the midbrain, there were significant differences in [^{3}H]GABA binding in the locus coeruleus and the nucleus tractus solitarius (see Figure 3B). In these regions, [^{3}H]GABA binding was two to threefold higher in the H animals, as compared with both NH and MS rats. In the nucleus tractus solitarius, [^{3}H]GABA binding in the MS rats was especially low, significantly lower than in either H or NH animals.

A similar, but more extensive, pattern of differences emerged for [^{3}H]flunitrazepam binding. In corticolimbic

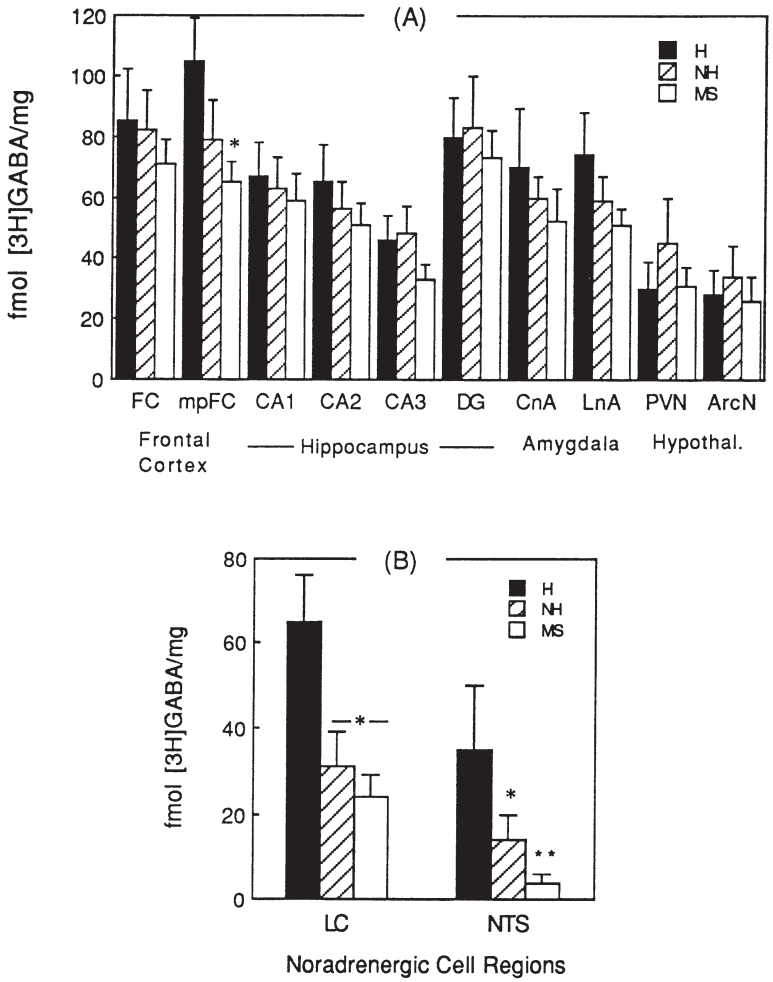

Figure 3. $GABA_A$ receptor binding (fmol [^{3}H]GABA binding per mg protein; mean ± SEM) in corticolimbic (upper panel) and noradrenergic cell body regions (lower panel) in handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 5–6 per group). Abbreviations: frontal cortex (FC), medial prefrontal cortex (mpFC), hippocampal regions of the Ammon's Horn (CA1-4), and dentate gyrus (DG), the central (CnA) and lateral (LnA) n. of the amygdala, the periventricular (PVN) and arcuate regions (ArcN) of the hypothalamus, the locus coeruleus (LC) and the n. tractus solitarius (NTS). **(A)** *$p < .05$ for comparison of H versus MS rats; **(B)** *$p < .05$ or **$p < .01$ for comparison of H versus NH/MS rats.

structures, there were significant differences in [^{3}H]flunitrazepam binding in the lateral and central nuclei of the amygdala (see Figure 4A). Within both regions, [^{3}H]flunitrazepam binding was significantly higher in H as compared with NH or MS animals. There were also significant differences in [^{3}H]flunitrazepam binding in the locus coeruleus, the C1 catecholamine cell body regions, and the nucleus tractus solitarius (see Figure 4B). Again, in the nucleus tractus solitarius, the [^{3}H]flunitrazepam binding in the MS rats was greatly reduced.

[^{3}H]flunitrazepam binding reflects both type I and II CBZ receptor types. To examine the relevant receptor subtype, we examined CBZ binding using [^{3}H]zolpidem, which selectively labels the type I receptor subtype (Ruano et al. 1993). The differences in [^{3}H]zolpidem binding paralleled those for [^{3}H]flunitrazepam. Thus, H animals showed increased [^{3}H]zolpidem binding in the central and lateral n. of the amygdala, as well as in the locus coeruleus and n. tractus solitarius (see Figure 5). In addition, using the more specific type I CBZ receptor ligand, we found decreased [^{3}H]zolpidem binding in the medial prefrontal cortex of the MS rats.

γ_2 In Situ Hybridization

The results of the γ_2 in situ hybridization study paralleled those of the CBZ receptor autoradiography. Thus, there was significantly higher levels of γ_2 mRNA expression in the lateral, basolateral, and central nuclei of the amygdala as well as in the locus coeruleus and the nucleus tractus solitarius in handled, as compared with either nonhandled or maternal separation rats (see Figure 6). There were no group differences in the CA1 region of the hippocampus; these findings again parallel the result of the receptor binding studies. Note, two forms of the γ_2 subunit are generated by alternative RNA splicing, resulting in the short (γ_{2s}) and long (γ_{2L}) variants (Whiting et al. 1990). Our probe was not selective; thus, we are unable to determine whether differences reflect greater levels of one variant or another.

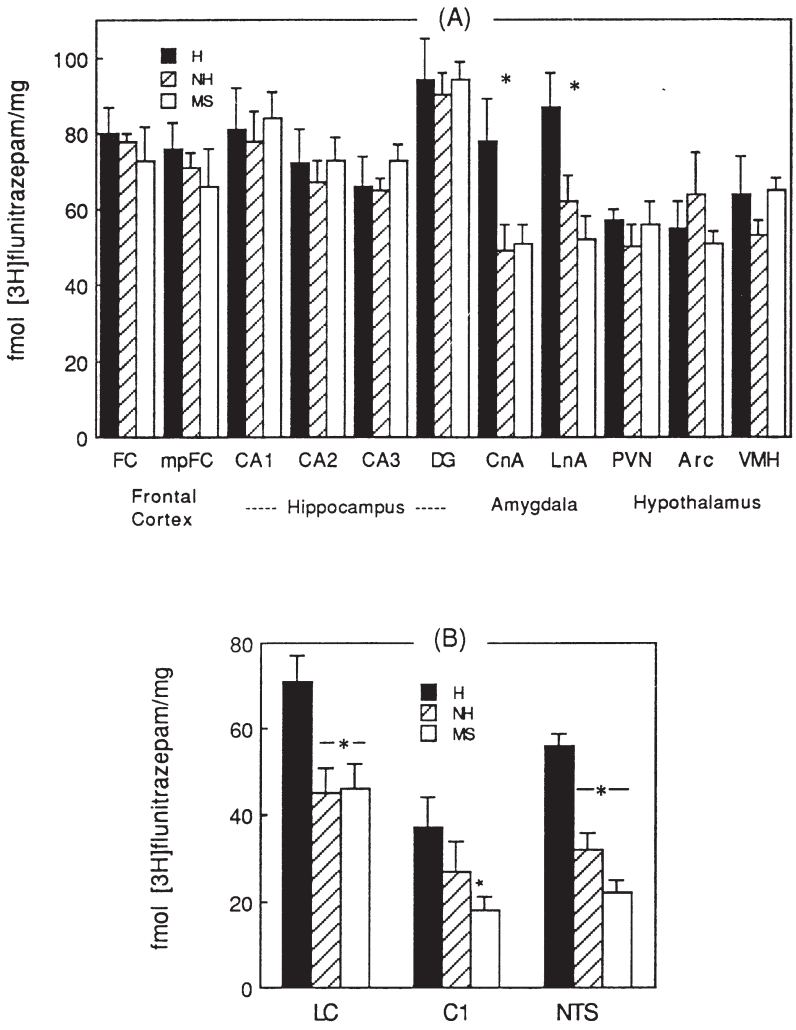

Figure 4. Central benzodiazepine receptor binding (fmol [^{3}H]flunitrazepam binding per mg protein; mean ± SEM) in corticolimbic (upper panel) and noradrenergic cell body regions (lower panel) in handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 5–6 per group). See Figure 3 legend for abbreviations. **(A)** * $p < .01$ for comparison of H versus NH/MS rats; **(B)** *$p <$ .01 for comparison with H rats.

DISCUSSION

The results of these studies confirm and extend previous research on the effects of early rearing conditions on behavioral responses to stress. The classical studies of Denenberg, Levine, and colleagues showed that postnatal handling decreased the expression of fear-related behaviors under stressful conditions (see Denenberg 1964; Levine 1975). More recent studies have shown that these effects are particularly notable under conditions of novelty (e.g., Bodnoff et al. 1987; Nunez et al. 1996; Escorihuela et al. 1992). In the present studies, differences in the expression of both appetitive and exploratory behaviors between H and NH or MS rats were apparent in response to novelty.

The results of a recent study (Plotsky et al. 1999) show that, in terms of HPA responses to stress, H rats are actually comparable to laboratory rats reared under standard conditions. Most breeding colonies perform cage cleaning repeatedly during the first 2 weeks of life, and this involves briefly removing the mother and the pups from the maternity cage. This procedure represents a variant of the handling procedure. Thus, the NH rats represent an interesting group, the absence of this manipulation results in increased fearfulness.

In recent studies, we have found evidence for the idea that the effects of handling on either endocrine (Liu et al. 1997) or behavioral/emotional (Caldji et al. 1998) responses to stress are mediated by handling-induced changes in maternal behavior (Smotherman and Bell 1980; Hennessy et al. 1982). Mothers of handled pups exhibit increased licking/grooming and arched-back nursing (Lee and Williams 1975; Liu et al. 1997; Caldji et al. 1998). Thus, handling seemss to exert its effects on the development of fear-related behaviors by ensuring a consistently high level of licking/grooming and arched-back nursing during early life. The importance of maternal care is also reflected in the effects of repeated periods of prolonged maternal separation, which produce increased fearfulness in response to novelty, as compared to the handling manipulation. Such effects are apparent in rhesus monkeys, where re-

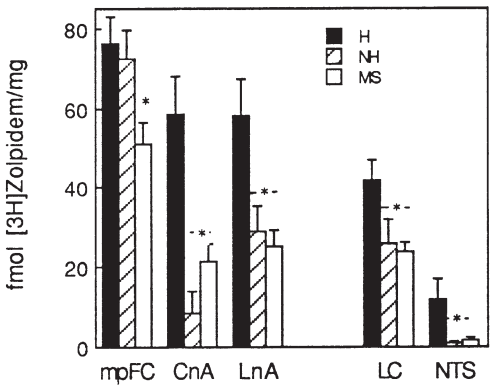

Figure 5. Type I central benzodiazepine receptor binding (fmol [^{3}H]zolpidem binding per mg protein; mean ± SEM) in corticolimbic (upper panel) and noradrenergic cell body regions (lower panel) in handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 5–6 per group). See Figure 4 legend for abbreviations. *$p < .01$ for comparison of H versus NH/MS rats.

peated periods of maternal separation resulted in increased fearfulness (e.g., Sackett 1969). Maternal separation in the rat, unlike handling, does not produce an increase in maternal licking/grooming or arched-back nursing; in fact, the opposite occurs. The offspring in the maternal separation condition experience long periods during which maternal care is absent. Moreover, even when the dam is present, the frequency of licking/grooming and arched-back nursing is reduced to levels typical of those observed in the mothers of NH litters. Taken together, these findings underscore the importance of maternal care in the development of fearfulness and provide an explanation for the comparable performance of the MS and NH animals.

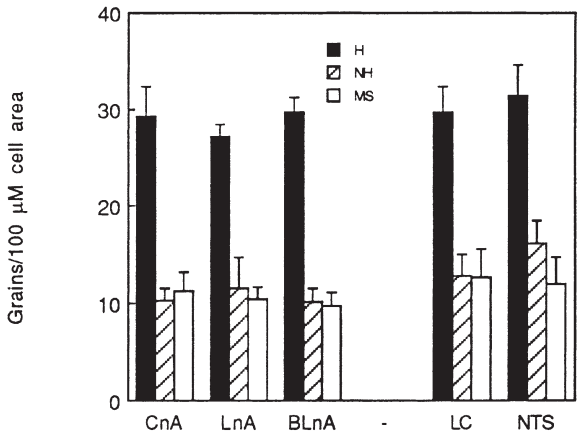

Figure 6. Mean ± SEM levels of expression of the mRNA for the γ_2 subunit of the $GABA_A$ receptor complex (number of grains per cell area) amygdaloid, and noradrenergic cell body regions in handled (H), nonhandled (NH), and maternal separation (MS) animals (n = 5–6 per group). Levels are significantly higher in H animals cas ompared with either NH or MS animals for each region shown, $p < .0001$. See Figure 3 legend for abbreviations.

These behavioral differences were associated with substantial effects of early experience on both $GABA_A$ and CBZ receptor levels. The CBZ site is a component of the $GABA_A$ receptor complex, and agonist binding to the CBZ site is associated with increased affinity of the $GABA_A$ receptor for GABA. CBZ activation is thought to enhance GABAergic inhibition of fear and anxiety. It is interesting that the differences observed in $GABA_A$ and CBZ receptor binding in corticolimbic structures were anatomically distinguishable. Thus, differences in $GABA_A$, but not CBZ binding were apparent in the frontal cortex; whereas, the opposite was true for differences in the lateral and central nuclei of the amygdala (see Figures 3 and 4). In contrast, in the locus coeruleus and the nucleus tractus solitarius MS and NH rats showed greatly reduced levels of both $GABA_A$ and CBZ receptors. In addition, levels of the mRNA encoding for the γ2 $GABA_A$ receptor subunit were significantly elevated in the amygdala, locus coeruleus, and nucleus tractus solitarius of H animals. The γ2 subunit seems to be essential for high affinity CBZ binding to the $GABA_A$ receptor complex (e.g., Pritchett et al. 1989). Interestingly, Caldji et al. (1998) found that the adult offspring of High Licking/Grooming and Arched-Back Nursing (High LG-ABN) mothers showed significantly increased $GABA_A$ and CBZ receptor binding in the amygdala as well as in the LC and NTS in comparison to the offspring of Low Licking/Grooming and Arched-Back Nursing (Low LG-ABN) mothers.

Although these data are correlational, there is evidence for the idea that differences in fearful responses to novelty could be associated with differences in $GABA_A$/CBZ receptor levels. Of particular interest are the results of human clinical studies. Glue et al. (1995) found that subjects that were high on measures of neuroticism showed reduced sensitivity to the benzodiazepine midazolam. In a series of studies, Roy-Byrne and colleagues (see Roy-Byrne et al. 1990, 1996) found reduced sensitivity to diazepam in patients with panic disorders and obsessive compulsive disorders and proposed that the reduced CBZ sensitivity was related to the phenomenon of anxiety. Although these studies have directly not shown decreased CBZ receptor levels, the findings are certainly consistent with the idea that decreased receptor levels in humans could be related to increased vulnerability to anxiety disorders. The hypothesis here is that there exists an endogenous anxiolytic ligand for the CBZ receptor and that decreased CBZ binding sites would, thus, result in enhanced fearfulness in the face of threat. The results of at least one study directly relate the effects of rearing condition on CBZ receptor levels with those on behavior. Escorihuela et al. (1992) found that postnatal handling im-

proved performance in a test of two-way avoidance task. The effect of handling was significantly reversed in animals treated acutely with the CBZ receptor antagonist, RO 15-1788.

We observed differences in $GABA_A$/CBZ receptor binding as a function of early rearing condition at a number of sites, including the lateral and central n. of the amygdala, the medial prefrontal cortex, as well as directly in the regions containing noradrenergic cell bodies. BZ agonists have been thought to exert anxiolytic effects via their actions at a number of limbic areas (e.g., Thomas et al. 1985; Gray 1987; Persold and Treit 1995; Gonzalez et al. 1996). To date, the evidence is perhaps strongest for BZ effects at the level of the lateral and central n. of the amygdala. Thus, the direct administration of drugs that enhance GABAergic activity via actions at BZ receptor sites into the amygdala yields an anxiolytic effect (Hodges et al. 1987). There is evidence for BZ action directly at the level of both the lateral and the central n. of the amygdala. The results of recent studies have indicated that the corticotropin-releasing factor (CRF) neurons within the amygdala might serve as a specific target for BZ effects. The BZ receptor agonist, alprazolam, has been shown to reduce CRF content in the locus coeruleus (Owens et al. 1991). The amygdala is a significant source of the CRF in the region of the locus coeruleus (Gray et al. 1989; Valentino 1990; Koegler-Muly et al. 1993; van Bockstaele et al. 1996). de Boer et al. (1992) have shown that BZ administration attenuates the effects of intracerebroventricular (ICV) CRF treatment, suggesting that the CRF system is a target for the anxiolytic effects of the BZs (also see Swerdlow et al. 1986). We have recently reported significantly elevated CRF mRNA in amygdala of MS and, to a lesser extent, NH rats as compared with H rats (Plotsky et al. 1999, submitted). The same pattern was observed for Corticotropin-Releasing Factor Immunoreactivity (CRFir) in the locus coeruleus (also see Ladd et al. 1996). Considering the importance of this amygdaloid CRF system in mediating behavioral responses to novelty (Hitchcock and Davis 1986; Liang et al. 1992; Krahn et al. 1988), we propose that BZ–CRF interactions within the amygdala serve as a critical neural substrate for the behavioral differences in response to novelty observed among H, NH, and MS rats.

In addition to the amygdala, there is also evidence for the importance of BZ action at the level of the frontal cortex (Lippa et al. 1979) for anxiolytic effects. It seems likely that a variety of structures are involved in mediating the anxiolytic effects of BZs, depending upon the nature of the stressor (see Gonzalez et al. 1996). In this respect, because we have found alterations at several sites, the differences in $GABA_A$/CBZ receptor binding as function of early rearing condition could serve to influence a wide range of behavioral and endocrine responses.

These findings are also of interest in light of the substantial differences in HPA responses to stress in H versus NH or MS rats (Plotsky and Meaney 1993). HPA responses to stress are mediated by the release of CRF from the paraventricular n. of the hypothalamus. Interestingly, we found no differences in $GABA_A$/CBZ receptor binding in the Paraventricular-Releasing Factor Immuno-reactivity (PVNh). However, there were highly significant differences in the noradrenergic cell body regions, including C1, the locus coeruleus, and the NTS. Noradrenergic input to the PVNh provides the major known source of activation for CRF synthesis and release (see Plotsky et al. 1989; Pacak et al. 1995). Thus, the increased $GABA_A$/CBZ receptor binding observed in these regions in the H rats may also serve to dampen HPA responses to stress by decreasing the magnitude of the noradrenergic signal.

Our findings are also interesting to consider in light of the known effects of BZs on responses to different forms of stress (see Malizia et al. 1995; O'Boyle et al. 1986; Goldstein et al. 1982). BZs are known to decrease autonomic, endocrine, and emotional responses to anticipatory forms of stress, where the threat is implied. In contrast, BZs do not affect responses to forms of stress that involve actual pain or tissue damage. A comparable distinction has emerged in studies of HPA responses to stress in H and NH rats. H rats show reduced plasma ACTH and corticosterone responses to novelty, air-puff startle, restraint, and human handling, but not to cold or to endotoxin injection (see Bhatnagar et al. 1995; Meaney et al. 1996). These findings lend further support for the idea that differences in CBZ receptor levels may contribute to the differences in behavioral and endocrine responses to stress observed among H, NH, and MS rats.

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