Neuropsychopharmacology (2003) 28, 1950–1959

www.neuropsychopharmacology.org

Variations in Maternal Care Alter $GABA_A$ Receptor Subunit Expression in Brain Regions Associated with Fear

Christian Caldji[1], Josie Diorio[1] and Michael J Meaney*[,1]

[1]*Developmental Neuroendocrinology laboratory, Douglas Hospital Research Centre, McGill University, Montréal, Canada H4H 1R3*

Maternal care influences the development of stress reactivity in the offspring. These effects are accompanied by changes in corticotropin-releasing factor (CRF) expression in brain regions that regulate responses to stress. However, such effects appear secondary to those involving systems that normally serve to inhibit CRF expression and release. Thus, maternal care over the first week of life alters $GABA_A$ (gamma-aminobutyric acid)$_A$ receptor mRNA subunit expression. The adult offspring of mothers that exhibit increased levels of pup licking/grooming and arched back-nursing (high LG-ABN mothers) show increased α1 mRNA levels in the medial prefrontal cortex, the hippocampus as well as the basolateral and central regions, of the amygdala and increased γ2 mRNA in the amygdala. Western blot analyses confirm these effects at the level of protein. In contrast, the offspring of low LG-ABN mothers showed increased levels of α3 and α4 subunit mRNAs. The results of an adoption study showed that the biological offspring of low LG-ABN mothers fostered shortly after birth to high LG-ABN dams showed the increased levels of both α1 and γ2 mRNA expression in the amygdala in comparison to peers fostered to other low LG-ABN mothers (the reverse was true for the biological offspring of high LG-ABN mothers). These findings are consistent with earlier reports of the effects of maternal care on $GABA_A$/benzodiazepine receptor binding and suggest that maternal care can permanently alter the subunit composition of the $GABA_A$ receptor complex in brain regions that regulate responses to stress.
Neuropsychopharmacology (2003) **28,** 1950–1959, advance online publication, 23 July 2003; doi:10.1038/sj.npp.1300237

Keywords: amygdala; rat; gene expression; $GABA_A$ receptor; benzodiazepine receptor; fear/anxiety

INTRODUCTION

Naturally occurring variations in maternal behavior in the rat directly influence the development of individual differences in stress reactivity (Liu *et al*, 1997; Caldji *et al*, 1998; Francis *et al*, 1999). Under conditions of stress, the adult offspring of mothers who showed an increased frequency of pup licking/grooming and arched-back nursing (high LG-ABN mothers), exhibit more modest pituitary–adrenal responses and decreased fearfulness compared with the offspring of low LG-ABN dams. Importantly, cross-fostering offspring from low-to-high LG-ABN mothers reverses the pattern of stress reactivity that is characteristic of the normal offspring of low LG-ABN mothers, and the reverse is also true (Francis *et al*, 1999). The increased stress reactivity of the adult offspring of low LG-ABN mothers is associated with elevated corticotropin-releasing factor (CRF) gene expression in both the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala (Diorio, Francis and Meaney, unpublished). CRF has been established as a mediator of behavioral and endocrine responses to stress, and this effect is, in part, mediated by CRF action at the level of the noradrenergic cell bodies in the locus coeruleus and the nucleus of the solitary tract (Butler *et al*, 1990; Valentino *et al*, 1998; Bakshi *et al*, 2000).

Maternal care alters benzodiazepine (BZ) receptor binding in the amygdala and locus coeruleus of the offspring such that among adult animals, BZ receptor levels in the central nucleus of the amygdala are highly correlated ($r = +0.87$) with the frequency of maternal licking/grooming in infancy (Caldji *et al*, 1998). The gamma-aminobutyric $acid_A$ ($GABA_A$) receptor system inhibits CRF activity, particularly at the level of the amygdala and locus coeruleus (Owens *et al*, 1991; Skelton *et al*, 2000). Predictably, behavioral responses to stress are inhibited by BZs, which exert their potent anxiolytic effect by enhancing GABA-mediated Cl^- currents through $GABA_A$ receptors (Barnard *et al*, 1988; Sieghart, 1995; McKernan and Whiting, 1997; Mehta and Ticku, 1999). BZ receptor agonists exert anxiolytic effects via their actions at a number of limbic areas, depending upon the test conditions. However, to date, the evidence is perhaps strongest for BZ effects at the level of the basolateral complex of the amygdala, comprising the lateral, basal and anterior basal nuclei (Pitkanen *et al*, 1997), and the central nucleus of the amygdala. Direct

*Correspondence: Dr MJ Meaney, Developmental Neuroendocrinology Lab., Douglas Hospital Research Centre, 6875 Boul. LaSalle, Montreal, Quebec, Canada H4H 1R3, Tel: +1 514 761 6131 ext 3938, Fax: +1 514 762 3034, E-mail: michael.meaney@mcgill.ca
Received 26 January 2003; revised 28 April 2003; accepted 02 May 2003
Online publication: 20 January 2003 at http://www.acnp.org/citations/Npp051902303 4/default.pdf

administration of BZs into the basolateral or central regions of the amygdala yields an anxiolytic effect (Hodges *et al*, 1987; Pesold and Treit, 1995; Gonzalez *et al*, 1996). Previous studies have found that the offspring of low LG-ABN mothers exhibit increased fearfulness in comparison to those of high LG-ABN dams (Caldji *et al*, 1998). In the current studies, we provide evidence for a profound influence of maternal behavior on $GABA_A$ receptor subunit gene expression that is most apparent in the basolateral and central nuclei of the amygdala, regions that are crucial for behavioral and autonomic expressions of fear (Schafe *et al*, 2001).

MATERIALS AND METHODS

Animals

The mothers were Long-Evans hooded rats obtained from Charles River Canada (St Constant, Qué bec) and housed in 46 cm × 18 cm × 30 cm 'Plexiglass' cages. Food and water were provided *ad libitum*. The colony was maintained on a 12 : 12 light : dark schedule with lights on at 08.00 h. The animals underwent routine cage maintenance beginning on Day 12, but were otherwise unmanipulated. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care with protocols approved by the McGill Committee on Animal Care.

The behavior of each dam was observed for eight, 60-min observation periods daily for the first 8 days postpartum (Myers *et al*, 1989; Liu *et al*, 1997; Caldji *et al*, 1998; Francis *et al*, 1999). Observers were trained, using video tapes and still photography, to a high level of inter-rater reliability (ie >0.90). Observations were performed at six periods during the light phase (08.00, 10.00, 11.00, 14.30, 16.00, and 18.00 h) and two periods during the dark phase of the L : D cycle (20.00 and 06.00 h). Within each observation period, the behavior of each mother was scored every 4 min (15 observations/period × eight periods per day = 120 observations/mother/day) for the following behaviors: mother off pups, mother carrying pup, mother licking and grooming any pup, mother nursing pups in either an arched-back posture, a 'blanket' posture in which the mother lays over the pups, or a passive posture in which the mother is lying either on her back or side while the pups nurse (see Myers *et al*, 1989; Liu *et al*, 1997; Caldji *et al*, 1998; Francis *et al*, 1999 for a description of behaviors).

The frequency of maternal licking/grooming and arched-back nursing across a large number of mothers is normally and not bi-modally distributed (Champagne *et al*, in press). Hence, the high and low LG-ABN mothers represent two ends of a continuum, rather than distinct populations. In order to define these populations for the current study, we observed the maternal behavior in cohorts of mothers, generally 30–40 dams with their pups, and devised the group mean and standard deviation for each behavior over the first 8 days of life as previously described (Liu *et al*, 1997; Caldji *et al*, 1998; Francis *et al*, 1999). High LG-ABN mothers were defined as females whose frequency scores for both licking/grooming and arched-back nursing were greater than 1 SD above the mean. Low LG-ABN mothers were defined as females whose frequency scores for both licking/grooming and arched-back nursing were greater than 1 SD below the mean.

At the time of weaning on Day 22 of life, the male offspring were housed in same-sex, same litter groups of three to four animals per cage until Day 45 of life, and two animals per cage from this point until the time of testing, which occurred no earlier than 100 days of age. All experiments were performed by individuals who were blind to the developmental history of the animals.

Adoption Study

The adoption study was performed using a limited cross-fostering design (McCarty and Lee, 1996; Francis *et al*, 1999) in which only two pups from each litter, one male and one female, were fostered onto other mothers in order to maintain the original gender distribution. The intention here was to avoid disturbances in maternal behavior than are known to be associated with the wholesale fostering of entire litters (Maccari *et al*, 1995). Within 6 h following the birth of the last pup, one male and one female were selected at random and placed into the host litter. The entire cross-fostering procedure was completed within 15 min of having originally disturbed the litters. Pups born to mothers previously characterized as high or low LG-ABN dams were used on the basis of previous findings showing that the frequency of maternal licking/grooming towards pups of the first litter is highly correlated ($r = +0.84$) with that directed towards pups of the second litter (Champagne *et al*, in press). Pups born to high LG-ABN mothers were then cross-fostered onto either another high LG-ABN mothers (high–high) or low LG-ABN mothers (high–low). Likewise, pups born to low LG-ABN mothers were then cross-fostered onto either other high LG-ABN mothers (low–high) or other low LG-ABN mothers (low–low). The results of a previous adoption study showed that the limited fostering does not alter the maternal behavior of the dam (Francis *et al*, 1999).

In Situ Hybridization

Brains were obtained by rapid decapitation shortly (<20 s) following the removal of the animal from the home cage. Brains were frozen in isopentane maintained on dry ice 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. The 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). They were then dehydrated using a 50–100% ethanol gradient, placed in chloroform for 10 min, followed by rehydration in 95% ethanol. The sections were then incubated overnight at 37°C with 75 μl/section of hybridization buffer containing 50% dionized 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 oligonucleotide probe. Oligonucleotide probes (see Table 1) were synthesized (Beckman 1000 DNA Synthesizer, Beckman, USA) and labeled using a DNA 3′-end labeling kit (Boehringer Mannheim, USA). Note that the γ2 oligonucleotide sequence

Table 1 List of Antisense Oligonucleotide Probes used in the *In Situ* Hybridization Studies and the Sources for the Sequences

$\alpha 1$	5′ GGG GTC ACC CCT GGC TAA GTT AGG GGT ATA GCT GGT TGC TGT AGG 3′ (Wisden *et al*, 1992)
$\alpha 2$	5′ CAA CGG CTA CAG CAG 3′ (Wisden *et al*, 1992)
$\alpha 3$	5′ CAC TGT TGG AGT TGA AGA AGC ACT GGG AGC AGC AGC AGC CTT GGA AGT 3′ (Poulter *et al*, 1992)
$\alpha 4$	5′ CAA GTC GCC AGG CAC AGG ACG TGC AGG AGG GCG AGG CTG ACC CCG 3′ (Wisden *et al*, 1992)
$\alpha 5$	5′ CCC AGT TGT AAA AGC ATT TGT TGA CTT ATT TAG TAT GAG TTC ACG TTC 3′ (Poulter *et al*, 1992)
$\beta 1$	5′ GAC TTT GTT CAT CTC CAG TTT GTT CTT TTC ATT GGC ACT CTG GTC TTG 3′ (Ma and Barker, 1995)
$\beta 2$	5′ TTT CCG ATA CTG GAT GCT GGA GGC ATC ATA GGC CAG CAT TGT GCT CCT TGG GTC TCC AAG 3′ (Ymer *et al*, 1989)
$\beta 3$	5′ CCC GTG AGC ATC CAC CCG GTT GAT TTC ACT CTT GGA TCG ATC ATT CTT 3′ (Ma and Barker, 1995)
$\gamma 1$	5′ GCC CTC CAA GCA CTG GTA ACC ATA ATC ATC TTC CCC TTG AGG CAT AGA 3′ (Ma and Barker, 1995)
$\gamma 2$	5′ GTC ATA GCC ATA TTC TTC ATC CCT CTC TTG AAG GTG GGT GGC 3′ (Wisden *et al*, 1992)

used in this study recognizes both γ2L and γ2S variants of the γ2 subunit. Preliminary studies using a scrambled versions of the α1, α2, and γ2 probes yielded no specific signal on brain sections (data not shown). Following hybridization, slides were washed 4×30 min in $1 \times$ SSC at 55°C, rinsed briefly in water, dried, and apposed to Hyperfilm for 21 days along with sections of ^{35}S-labeled standards prepared with known amounts of ^{35}S in a brain paste. The hybridization signal within various brain regions was quantified using densitometry with an image analysis system (MCID, St Catherines, Ontario). The data are presented as arbitrary optical density (absorbance) units following correction for background and are the averages drawn from three sections per brain region/per animal. The anatomical level of analysis was verified using the rat brain atlas of Paxinos and Watson (1982) with Nissl-staining of sections following autoradiography.

Western Blotting

Following rapid decapitation, brains were removed and placed on ice. The amygdala were dissected, snap-frozen on dry ice, and stored at −80°C. Frozen samples were placed into a microcentrifuge tube containing ~5 volume of ice-cold sucrose buffer (320 mM sucrose/4 mM Hepes, pH 7.4) and homogenized in the same buffer using a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1100*g* and the supernatant was centrifuged for 15 min at 9200*g*. The resulting pellet was then resuspended in sucrose buffer (with 9 vol of ice-cold dH_2O), and homogenized with a Dounce homogenizer. Hepes (1 M, pH 7.4) was added to a final concentration (7.5 mM) and incubated on ice (30 min). The homogenate was centrifuged for 20 min at 25 500*g* and the pellet was discarded. The supernatant was centrifuged (48 000 rpm, 2 h), and the resulting pellet resuspended in 1 ml of buffer (sucrose (30 mM)/Hepes (4 mM), pH 7.4), and homogenized using a 25-gauge needle. Aliquots of the homogenates were taken to determine the levels of protein using the method of Bradford (1976) and ranged from 1.5 to 2.5 μg/μl.

Protein samples (25 μg) were mixed with an equal volume of $2\times$ sample buffer (0.25 M Tris-HCl, 20% glycerol, 4% SDS, 0.005% bromoethanol blue, and 5% β-mercaptoethanol) and subjected to denaturing and reducing conditions prior to separation using electrophoresis with Novex 4–12% Tris-glycine PAGE precast gels (Helixx Technologies, Scarborough, Ontario, Canada) with stained molecular markers loaded for reference (SeeBlue, Santa Cruz Biotech, CA, USA). Proteins were electrophoretically transferred according to the method of Towbin onto nitrocellulose membranes (Amersham, Oakville, Ontario) and air-dried overnight. The membranes were blocked for 1 h at room temperature with 5% Carnation dried milk in TBS-T (Tris, NaCl, 0.1% Tween-20, pH 7.6), washed briefly in TBS-T and incubated overnight at 4°C with antibodies to the α1, α2, or γ2 subunits of the $GABA_A$ receptor. All antibodies were generously provided by Dr Ruth McKernan (Merck Sharpe Dohme, Harlow, UK) and used at concentrations of 1 : 1000 (McKernan *et al*, 1991). Membranes were washed for 20 min with TBS-T and incubated with secondary horseradish peroxidase-labeled antibody (anti-rabbit Igg, Amersham Inc.) diluted 1 : 5000 in TBS-T for 1 h at room temperature. After 4×15 min washes in TBS-T, the membranes were then exposed using an ECL kit (Amersham) and apposed to film (ECL Hyperfilm, Amersham). In order to verify the accuracy of sample loading, membranes were stripped and reprobed with an α-tubulin monoclonal antibody (Biodesign International, Kennebunkport, ME) diluted at 1 : 5000. Optical density readings for bands were determined using a computer-assisted densitometry system (MCID Systems; St Catherines, Ontario).

Statistical Analysis

For each experiment, there were between one and two pups per litter representing at least three to a maximum of five litters per group. In order to control for any potential 'litter' effects, we analyzed the data both by subject and by litter. There were no differences between these two methods of analyses in the statistical outcomes for any of the experiments. For the sake of brevity, we provide the results of the analysis by subject. The *in situ* hybridization data were analyzed using a two-way (Group × Region) analysis of variance with Tukey *post hoc* tests where appropriate. The results of the Western blots were analyzed using *t*-tests for simple main effects of maternal care.

RESULTS

α Subunit mRNA Expression

There was a significant Group ($F = 13.22$; $df = 1,9$; $P < 0.01$) effect for α1 mRNA expression (Figure 1) reflecting the widespread differences across multiple regions. *Post hoc*

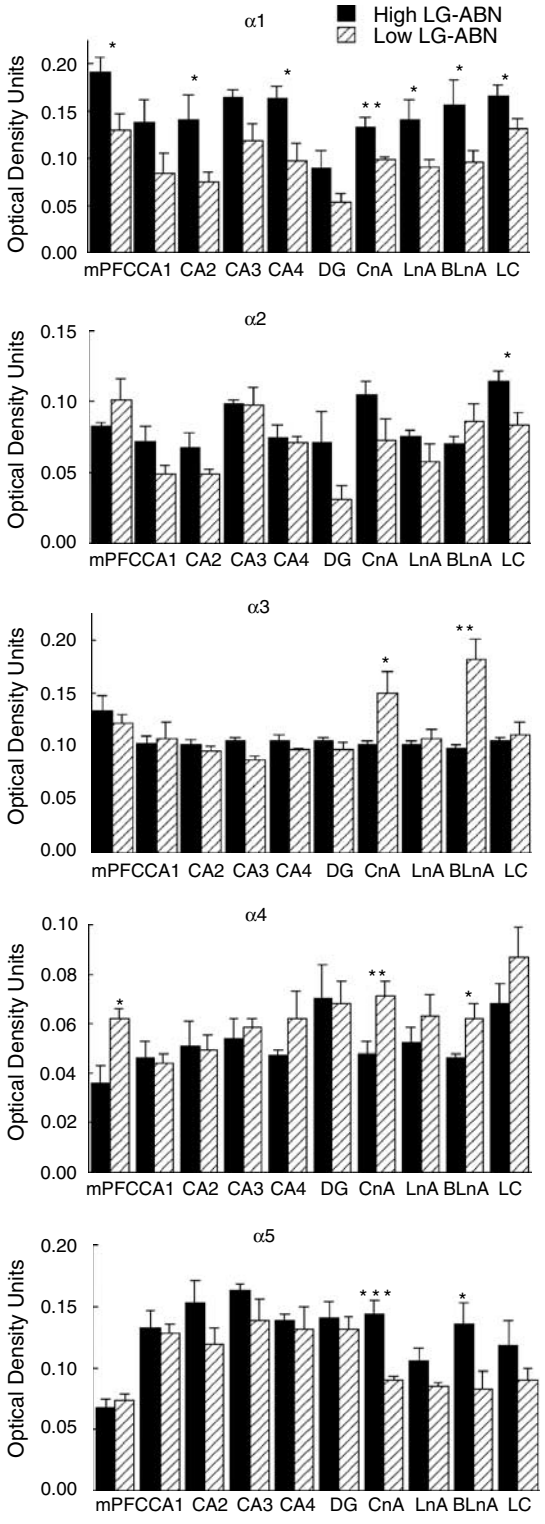

Figure 1 Mean ± SEM levels of mRNA for various α subunits of the $GABA_A$ receptor in various brain regions expressed as optical density units from autoradiograms from *in situ* hybridization studies with a ^{35}S-labeled oligonucleotide probes (see Table 1; $n=5$ animals per group). Abbreviations: medial prefrontal cortex (mPFC), dentate gyrus (DG), hippocampal Ammon's horn (CA1-4), basolateral nucleus of the amygdala (BLA), lateral nucleus of the amygdala (LA), central nucleus of the amygdala (CA), locus coeruleus (LC), nucleus of the solitary tract (NTS), thalamus (Thal), parietal cortex (PCtx). ***$P<0.01$; **$P<0.01$; *$P<0.05$.

analysis revealed increased α1 mRNA levels in the offspring of the high LG-ABN mothers in the prefrontal cortex, the CA2 and CA4 regions of the hippocampus, the central, lateral and basolateral nuclei of the amygdala, and the locus coeruleus. Analysis of the α2 mRNA data revealed neither a significant group nor a Group × Regions interaction effect. In general, levels of α2 mRNA expression were comparable between groups. There was a significant Group × Region interaction ($F=5.73$; $df=8,72$; $P<0.0001$) for α3 subunit mRNA expression. In contrast with the α1 expression pattern, α3 mRNA levels were significantly increased in the offspring of low LG-ABN mothers in the central and especially the basolateral nucleus of the amygdala. Likewise, there was a Group × Region interaction that approached significance ($F=1.96$; $df=8,72$; $P<0.10$) for α4 subunit mRNA expression and *post hoc* analysis showed increased levels of the mRNA levels for the α4 subunit in the central and basolateral nuclei of the amygdala, as well as in the prefrontal cortex of the offspring of low LG-ABN mothers. There was only a marginally significant Group × Region effect for levels of α5 subunit mRNA ($F=1.73$; $df=8,72$; $P<0.15$), although mRNA levels were significantly higher in the central and basolateral nuclei of the amygdala in the offspring of high compared with low LG-ABN mothers. Owing to the absence of the interaction effects, these latter findings should be viewed with caution. No other comparisons approached significance. There were no group difference in mRNA levels in the parietal cortex, hypothalamus, or thalamus for any of the α subunits.

β Subunit mRNA Expression

There was no significant Group × Region interaction for β1 subunit mRNA expression and no pattern of group differences was evident (Figure 2). β1 mRNA levels were significantly higher in the thalamus in the offspring of low LG-ABN mothers (data not shown). This was the only instance in which significant group differences in the mRNA levels for the $GABA_A$ receptor subunits were found in this region. There were no significant differences in the hippocampal/amygdaloid regions. Differences in β2 mRNA levels were regionally very specific, generally mapping onto the differences observed for the α1 subunit mRNA, and yielding a significant Group × Region interaction effect ($F=7.72$; $df=8,72$; $P<0.001$). β2 mRNA levels in the offspring of high LG-ABN mothers were significantly higher in the basolateral and central nuclei of the amygdala as well as the locus coeruleus (Figure 2). Levels of β3 mRNA were consistently higher in the offspring of high LG-ABN mothers yielding significant Group and Group × Region interaction effects ($F=2.52$; $df=8,72$; $P<0.05$). *Post hoc* analyses revealed that in the several regions of the hippocampus and amygdala, β3 mRNA levels were significantly in the offspring of high LG-ABN mothers. There were no group differences in mRNA levels in the parietal cortex or hypothalamus for any of the β subunits.

γ Subunit mRNA Expression

The pattern of group differences in the expression of the γ subunits was strikingly unique to the amygdala. Thus, there were significant Group × Region interaction effects in

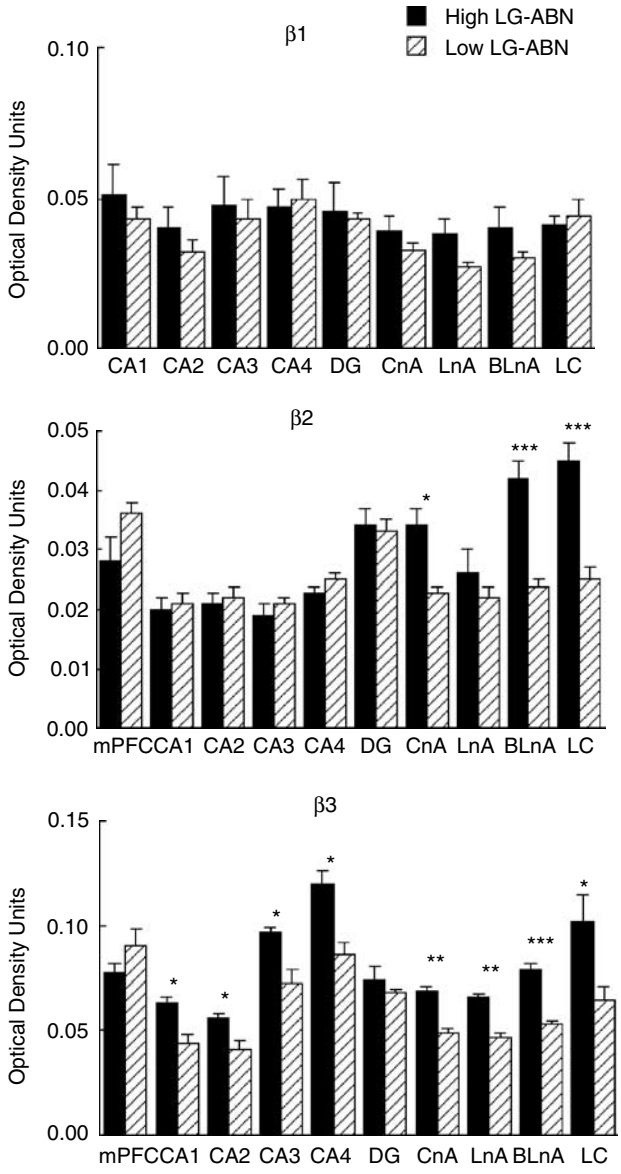

Figure 2 Mean ± SEM levels of levels of mRNA for various β subunits of the $GABA_A$ receptor in various brain regions expressed as optical density units from autoradiograms from *in situ* hybridization studies with a ^{35}S-labeled oligonucleotide probes (see Table 1; $n = 5$ animals per group). Abbreviations described in the caption to Figure 1. ***$P < 0.01$; **$P < 0.01$; *$P < 0.05$. Note that the signal in the mPFC was too low for reliable quantification.

mRNA levels for both the $\gamma 1$ ($F = 235$; $df = 8,72$; $P < 0.05$) and $\gamma 2$ ($F = 2.69$; $df = 8,72$; $P < 0.01$) subunits (see Figures 3a). *Post hoc* analysis revealed significantly increased levels of $\gamma 1$ and $\gamma 2$ mRNA levels in the central, basolateral, and lateral nuclei of the amygdala in the offspring of high compared with low LG-ABN mothers. In no other region were these differences significant. Figure 3b provides a photomicrograph of an autoradiogram revealing the differences in $\gamma 2$ mRNA levels in the central, basolateral, and lateral nuclei of the amygdala as a function of postnatal maternal care. Note the increased density of the signal in these regions in comparison to the neighboring corticomedial areas. There were no group differences in mRNA levels in the parietal cortex, hypothalamus, or thalamus for any of the γ subunits.

Western Blotting

In order to at least partially verify the functional significance of the mRNA studies, we compared $GABA_A$ receptor subunit-like immunoreactivity in amygdaloid samples from adult offspring of high or low LG-ABN mothers using Western blotting (see Figure 4). We focused on two subunits that provided the most reliable differences in mRNA, $\alpha 1$ and $\gamma 2$ subunits, as well as a negative control, the $\alpha 2$ subunit. The results reveal significant group differences in $\alpha 1$- and $\gamma 2$-like immunoreactivity ($t = 4.10$; $P < 0.01$; $t = 5.69$; $P < 0.005$, respectively), with no group difference in the levels of $\alpha 2$-like immunoreactivity. Bearing in mind the limitations with such comparisons, the group differences at the level of protein ($\sim$100–150%) exceeded those for mRNA ($\sim$30–80% differences depending upon the region).

Adoption Study

In the adoption study, we analyzed the expression of mRNAs for the $\alpha 1$ and $\gamma 2$ subunits focusing on the basolateral, central, and lateral regions of the amygdala. The logic here was to again focus on those subunits that provided the most reliable differences in the *in situ* hybridization study. In both cases, the results revealed highly significant effects for rearing mothering, but not biological mothering (see Figure 5). This pattern was striking for both the $\alpha 1$ (rearing mother: $F = 31.60$; 1,12; $P < 0.0001$. biological mother: $F < 1.0$; NS) and $\gamma 2$ (rearing mother: $F = 15.88$; 1,12; $P < 0.002$. biological mother: $F < 1.0$; NS) subunits in all regions, save for the $\gamma 2$ subunit mRNA levels in the lateral nucleus of the amygdala. Even here, however, the effect of the rearing mother was significant. There was no significant main effect of the biological mother for either subunit in any region examined. Thus, expression of the mRNAs for both the $\alpha 1$ and $\gamma 2$ subunits in animals born to low LG-ABn mothers, but reared by high LG-ABN dams was indistinguishable from that of the normal offspring of high LG-ABN mothers. The reverse was also true for pups born to high LG-ABN dams, but reared by low LG-ABn mothers.

DISCUSSION

Neuronal inhibition is mediated through GABA-gated Cl^- channels, collectively known as $GABA_A$ receptors. The $GABA_A$ receptor complex in the rat brain, which often includes a 'BZ' binding site, is most commonly arranged in a pentameric structure comprised of α, β, and γ subunits, in the form of two α, two β, and one γ subunit or of two α, one β, and two γ subunits (Barnard *et al*, 1988; Sieghart, 1995; McKernan and Whiting, 1997; Mehta and Ticku, 1999). The α subunit forms the GABA-binding site and the interface between the α and γ subunits appears to form the BZ receptor site (Khan *et al*, 1996). $GABA_A$ receptor activity, defined by Cl^- influx and changes in neuronal potential, is allosterically regulated by compounds acting at BZ receptor sites (Braestrup *et al*, 1984; Im and Blakeman, 1991; Maksay, 1993; Haefely, 1994). Interestingly, dynamic

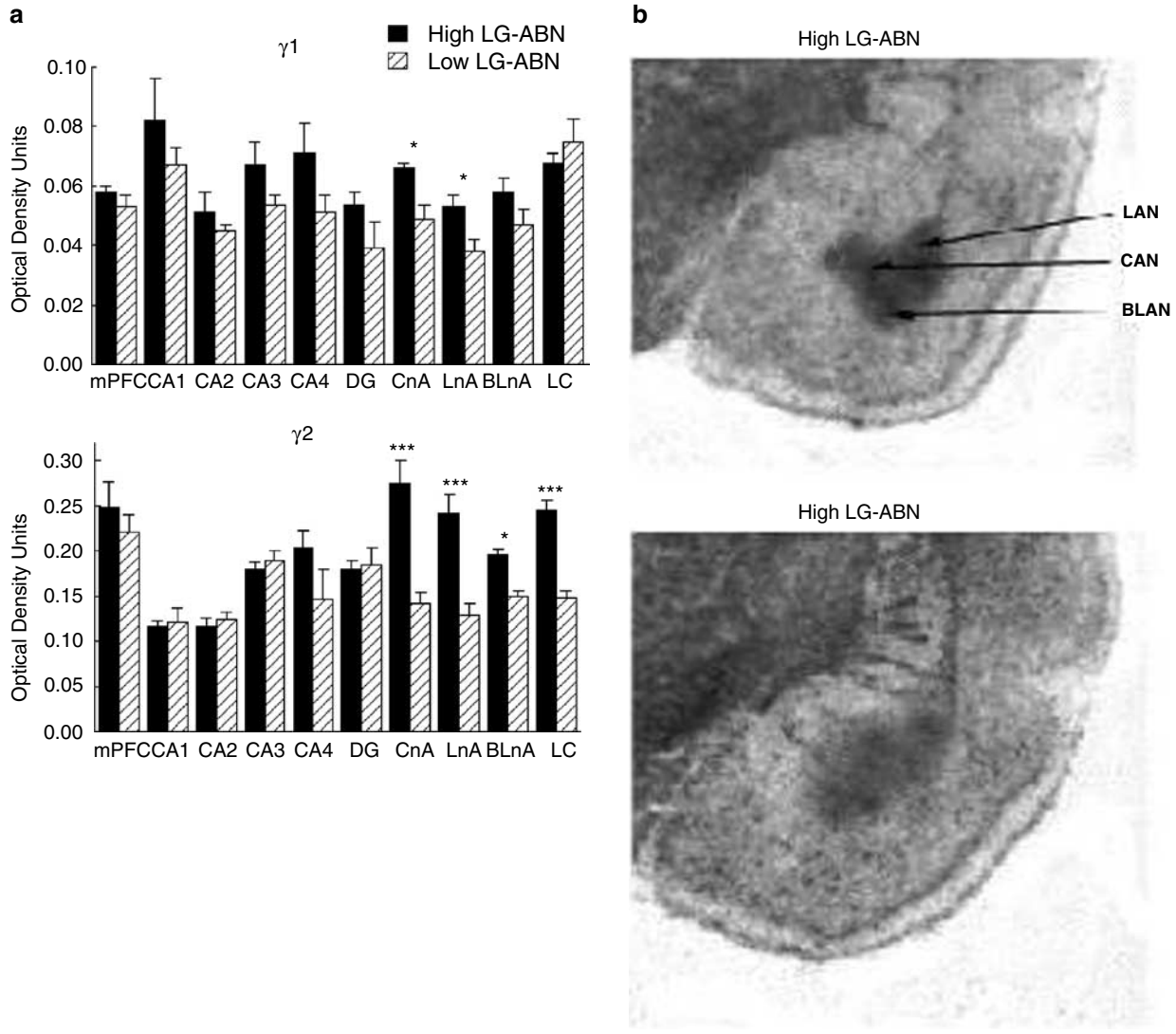

Figure 3 (a) Mean ± SEM levels of mRNA for various γ subunits of the GABA$_A$ receptor in various brain regions expressed as optical density units from autoradiograms from *in situ* hybridization studies with a ^{35}S-labeled oligonucleotide probes (see Table 1; $n = 5$ animals per group). Abbreviations described in the caption to Figure 1. ***$P < 0.01$; **$P < 0.01$; *$P < 0.05$. (b) Photomicrograph of a representative autoradiogram from the *in situ* hybridization study comparing adult offspring of high and low LG-ABN mothers. The photo is focused on the amygdaloid region. Note the higher density signal in the lateral, nasolateral, and central regions of the amygdala in the high LG-ABN offspring. Abbreviations as described in the caption to Figure 1.

variations in GABA$_A$ receptor function often occur as a result of such allosteric modulation of the GABA$_A$ receptor. Hence it seems reasonable to assume that the source of stable individual differences in GABA$_A$ receptor function might lie in variations in the expression of subunits that define BZ receptor binding.

Our results suggest that variations in maternal care permanently alter the subunit composition of the GABA$_A$ receptor complex in the offspring. Importantly, the results of the adoption study suggest that differences in GABA$_A$ receptor subunit expression were directly related to variations in maternal care (Figure 5). We found significantly increased levels of the mRNAs for the $\gamma 1$ and $\gamma 2$ subunits in the offspring of high compared with low LG-ABN mothers and the effect was strikingly unique to the amygdala. Differences in α subunit expression were more promiscuous. Levels of $\alpha 1$ mRNA were significantly higher in the amygdala, prefrontal cortex, hippocampus, and locus coeruleus of high compared with low LG-ABN offspring. The results of Western blotting studies for the $\alpha 1$ and $\gamma 2$ subunits suggest that the effects at the level of mRNA expression are reflected in differences in protein levels. Likewise, there were no group differences in $\alpha 2$ subunit expression in the amygdala at either the level of mRNA or protein. Interestingly, the adult offspring of the low LG-ABN mothers show *increased* expression of the mRNAs for the $\alpha 3$ and, to a lesser extent, the $\alpha 4$ subunit. As with the γ subunits, such differences were largely limited to regions of the amygdala. The only substantial difference in the levels of mRNA encoding for the β subunits was in $\beta 2$ mRNA in the amygdala and in $\beta 3$ subunit mRNA levels that was more broadly apparent. What makes this finding intriguing is that in native GABA$_A$ receptor complexes, the $\beta 2$ subunit is primarily associated with the $\alpha 1$ and $\gamma 2$ subunits and each of these subunits is encoded by genes located on chromosome 5 (5q34–q35) (Wilcox *et al*, 1992).

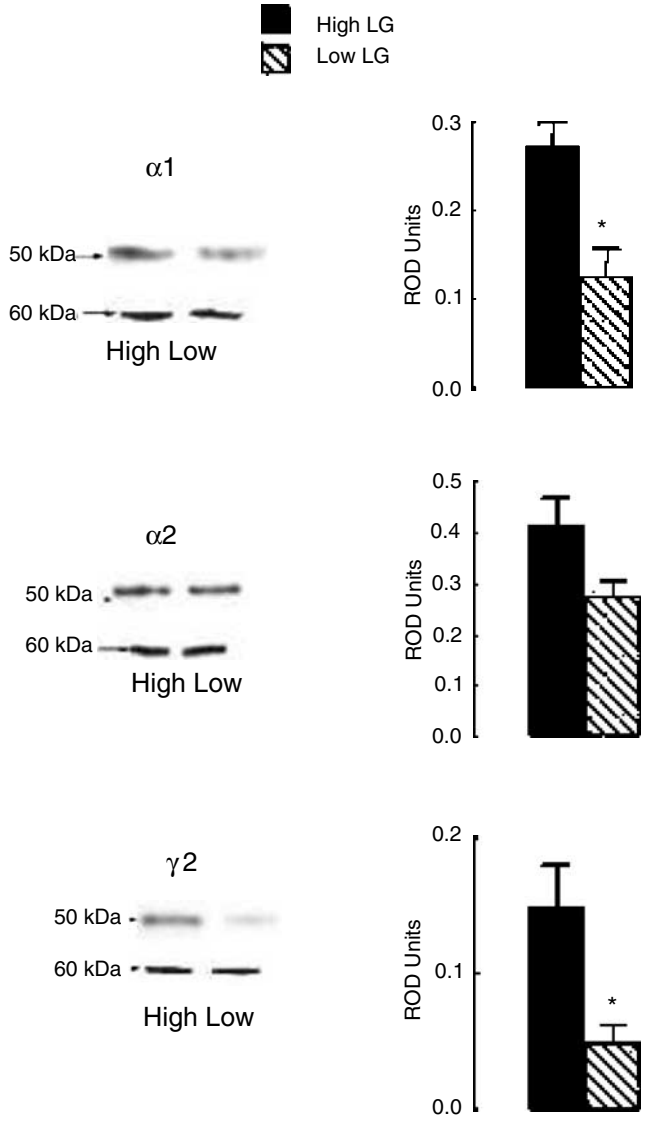

Figure 4 Mean ± SEM levels of α1, α2, and γ2 immunoreactivities on Western blots in amygdala samples from adult offspring high or low LG-ABN mothers ($n = 3$–4/group). $^*P < 0.01$. The left-hand side panels depict representatives blots for each subunit (top bands) along with bands for β-actin immunoreactivity from the same membranes used to control for loading errors.

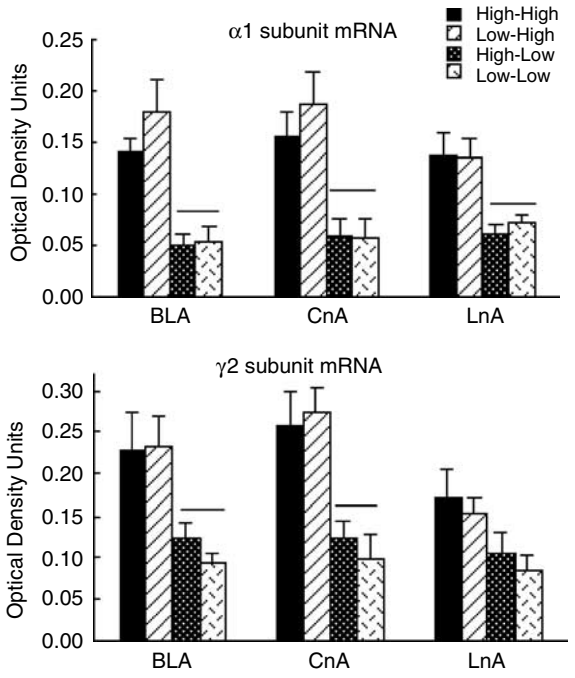

Figure 5 Mean ± SEM levels of mRNA for α1 and γ2 subunits of the $GABA_A$ receptor in the amygdala expressed as optical density units from autoradiograms from *in situ* hybridization studies with a ^{35}S-labeled oligonucleotide probes ($n = 4$–5 animals per group) in the biological offspring of high LG-ABN mothers cross-fostered to either high (high–high) or low (high–low) LG-ABN mothers and the biological offspring of low LG-ABN mothers cross-fostered to either high (low–high) or low (low–low) LG-ABN mothers. Abbreviations as described in the caption to Figure 1. $^{***}P < 0.01$; $^{**}P < 0.01$; $^*P < 0.05$.

The differences in subunit expression are likely to be of considerable importance for $GABA_A$ receptor pharmacology. Indeed, the adult offspring of high and low LG-ABN mothers differ in BZ receptor binding in the amygdala (Caldji *et al*, 1998). In this study, the correlation between maternal licking/grooming over the first 8 days of life and the adult level of BZ receptor binding in the central nucleus of the amygdala was +0.87. The inclusion of a γ1 or γ2 subunit in the $GABA_A$ receptor complex appears to define the presence of a BZ receptor site. Point mutations have been identified within both subunits that are sufficient to eliminate BZ receptor binding (Amin *et al*, 1997; Buhr and Sigel, 1997; Buhr *et al*, 1997; Wingrove *et al*, 1997) and animals bearing a null mutation of the γ2 subunit show approximately an 85% loss of [^{3}H]flunitrazepam binding (Gunther *et al*, 1995). Thus, the effect of maternal care on the expression of the γ subunits might well contribute to the previously observed difference in BZ receptor-binding capacity between high and low LG-ABN mothers (Caldji *et al*, 1998). Interestingly, in this earlier study differences in [^{3}H]flunitrazepam binding were largely unique to the amygdala, a finding that is similar to that for the γ1 or γ2 subunit mRNAs.

While the γ subunits define the presence of a BZ receptor, it is the α subunits that define the nature of the BZ receptor subtype (Barnard *et al*, 1988; Pritchett and Seeburg, 1991; Hadingham *et al*, 1993; McKernan and Whiting, 1997; Rudolph *et al*, 1999; Griebel *et al*, 2000). Receptors containing the α1 subunit exhibit BZ_1 receptor pharmacology, with a high affinity for classical BZ agonists, such as diazepam and alprazolam, and also for the atypical BZ agonists, CL218872 and zolpidem. Transfection studies of recombinant $GABA_A$ receptors comprised of α1, β, γ1, or γ2 subunits exhibit BZ_1 receptor pharmacology, with high-affinity binding for zolpidem (Barnard *et al*, 1988; Mehta and Ticku, 1999), which binds BZ_1 receptors with an affinity ($k_d \sim 7$ nM) that is at least 20 times greater than for BZ_2 receptors (Arbilla *et al*, 1986). Receptors composed of the α2, α3, or α5 subunits show BZ_2 receptor profiles, with high affinity for classical BZ agonists, but greatly reduced affinity for zolpidem. Thus, $GABA_A$ receptors composed of the α1, α2, α3, and α5 subunits are indistinguishable in their affinity for ligands, such as flunitrazepam, which bind with equal affinity to BZ_1 and BZ_2 receptors (see Barnard *et al*, 1988; Hadingham *et al*, 1993; McKernan and Whiting, 1997;

Mehta and Ticku, 1999 for reviews). $GABA_A$ receptors comprised of any of these subunits or with the $\alpha 4$ subunit are also indistinguishable in their affinity for the partial inverse agonist RO15-4513; note, however, receptors bearing the $\alpha 4$ subunit do not bind BZ agonists (Yang *et al*, 1995). Moreover, $GABA_A$ receptors comprised of the $\alpha 4$ subunit show an increased affinity for full inverse agonists, such as the β-carbolines (Yang *et al*, 1995). Finally, there is also a modest contribution of the β subunits to BZ receptor pharmacology: $GABA_A$ receptors comprised of either the $\beta 2$ or $\beta 3$ subunit show a two-fold higher affinity for zolpidem compared with those bearing a $\beta 1$ subunit. The mRNA levels for both the $\beta 2$ and $\beta 3$ subunit were higher in the offspring of high LG-ABN mothers, but again, only within the amygdala. These findings further underscore the apparent shift towards BZ_1 receptor expression in the amygdala in the offspring of high LG-ABN mothers. Prolonged periods of maternal separation in early life decreased the levels of both $\alpha 1$ (Caldji, Diorio, Plotsky and Meaney, unpublished) and $\gamma 2$ (Caldji *et al*, 2000) subunit mRNA. These effects on subunit expression were associated with differences in flunitrazepam binding, but such differences were substantially less impressive than those obtained using [^{3}H]zolpidem, a selective ligand for the BZ_1 receptor (Caldji *et al*, 2000).

BZ receptor agonists exert anxiolytic effects via their actions at a number of limbic areas, including the amygdala (Hodges *et al*, 1987; Pesold and Treit, 1995; Gonzalez *et al*, 1996). More recent studies have focused on the effects of intra-amygdaloid infusion of either $GABA_A$ or BZ receptor agonists or antagonists on fear conditioning. The results demonstrate potent effects of such treatments on the acquisition (amnesic effects) and expression (anxiolytic effects) of conditioned fear responses and underscore the importance of the amygdala as a critical target for the effects of $GABA_A$ and BZ receptor agonists (Brioni *et al*, 1989; Tomaz *et al*, 1993; Helmstetter and Bellgowan, 1994; Dickinson-Anson and McGaugh, 1997; Muller *et al*, 1997; Da Cunha *et al*, 1999). While maternal care altered subunit expression in the hippocampus and prefrontal cortex, the most consistent effects were clearly at the level of the basolateral and central regions of the amygdala. Predictably, the adult offspring of low LG-ABN mothers consistently show evidence for increased fearfulness in comparison to those of high LG-ABN mothers (Caldji *et al*, 1998; Francis *et al*, 1999), and these differences are reversed with cross-fostering (Francis *et al*, 1999).

The effect of maternal care on $GABA_A$ receptor subunit expression may provide a mechanism for the well-established relationship between early life events and vulnerability for anxiety disorders. Surprisingly, only a minority ($\sim$30%) of humans subjected to even profound trauma develop PTSD (Ressnick *et al*, 1993). The quality of early family life serves as a highly significant predictor of vulnerability to PTSD (Udwin *et al*, 2000) and significantly influences the risk for anxiety disorders (Tweed *et al*, 1989; Stein *et al*, 1996; Pruessner *et al*, 2000). Emotionally adverse stimuli activate the human amygdala (Cahill and McGaugh, 1998). Indeed, the degree of amygdaloid activation is highly correlated ($r = +0.93$) with recall of emotionally disturbing, but not neutral material. With high temporal resolution fMRI techniques, LaBar *et al* (1998) found increased amygdala activity during the acquisition phase of fear conditioning, and patients with amygdala damage show profound deficits in fear conditioning (Bechara *et al*, 1995; LaBar *et al*, 1995). Interestingly, individual differences in the degree of amygdaloid activation occurring during fear conditioning was highly correlated with the strength of a conditioned autonomic fear response (Furmark *et al*, 1997).

Studies in humans support the idea that alterations in the $GABA_A$/BZ receptor complex might form the basis of a vulnerability for anxiety disorders (Gorman *et al*, 2000). Unmedicated patients with a history of panic disorder show a significant decrease in labeling of the BZ receptor antagonist [^{11}C]flumazenil in the orbitoprefrontal cortex and amygdala/hippocampal region in PET studies (Malizia *et al*, 1998). The findings are consistent with those of pharmacological measures of BZ receptor sensitivity. Subjects high on measures of neuroticism show reduced sensitivity to the BZ receptor agonist, midazolam (Glue *et al*, 1995). Roy-Byrne *et al* (1990, 1996) found reduced sensitivity to diazepam in patients with panic disorders, and proposed that the reduced BZ receptor sensitivity was related to anxiety. Patients with panic attacks or high levels of general anxiety show decreased sensitivity to BZ-induced amnesia, sedation, and dampening of noradrenergic function compared with controls (Melo de Paula, 1977; Oblowitz and Robins, 1983). These findings suggest that early life events might alter the development of the $GABA_A$ receptor system in brain regions that mediate stress reactivity, and thus contribute to individual differences in vulnerability to anxiety disorders (Gorman *et al*, 2000).

ACKNOWLEDGEMENTS

This research was supported by a grant from the Canadian Institutes of Health Research to MJM. CC is a graduate fellow and a recipient of a Canadian Institutes of Health Research Doctoral Research Award. MJM was supported by a Senior Scientist award from the Canadian Institutes of Health Research and an NARSAD Distinguished Investigator award.

REFERENCES

Amin J, Brooks-Kayal A, Weiss DS (1997). Two tyrosine residues on the alpha subunit are crucial for benzodiazepine binding and allosteric modulation of gamma-aminobutyric acidA receptors. *Mol Pharmacol* **51**: 833–841.

Arbilla S, Allen J, Wick A, Langer SZ (1986). High affinity [^{3}H]zolpidem binding in the rat brain: an imidazopyridine with agonist properties at central benzodiazepine receptors. *Eur J Pharmacol* **130**: 257–263.

Bakshi VP, Shelton SE, Kalin NH (2000). Neurobiological correlates of defensive behaviors. *Prog Brain Res* **122**: 105–115.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G *et al* (1988). International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* **50**: 291–313.

Bechara A, Tranel D, Damasio H, Adolphs R, Rockland C, Damasio AR (1995). Double dissociation of conditioning and declarative knowledge relative to the amygdala and hippocampus in humans. *Science* **269**: 1115–1118.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein–dye binding. *Anal Biochem* **72**: 248–254.

Braestrup C, Honore T, Nielsen M, Petersen EN (1984). Ligands for benzodiazepine receptors with positive and negative efficacy. *Biochem Pharmacol* **33**: 859–862.

Brioni JD, Nagahara AH, McGaugh JL (1989). Involvement of the amygdala GABAergic system in the modulation of memory storage. *Brain Res* **487**: 105–112.

Buhr A, Schaerer MT, Baur R, Sigel E (1997). Residues at positions 206 and 209 of the alpha1 subunit of gamma-aminobutyric acidA receptors influence affinities for benzodiazepine binding site ligands. *Mol Pharmacol* **5**: 676–682.

Buhr A, Sigel E (1997). A point mutation in the gamma2 subunit of gamma-aminobutyric acid type A receptors results in altered benzodiazepine binding site specificity. *Proc Natl Acad Sci USA* **94**: 8824–8829.

Butler PD, Weiss JM, Stout JC, Nemeroff CB (1990). Corticotropin-releasing factor produces fear-enhancing and behavioural activating effects following infusion into the locus coeruleus. *J Neurosci* **10**: 176–183.

Cahill L, McGaugh JL (1998). Mechanisms of emotional arousal and lasting declarative memory. *Trends Neurosci* **21**: 294–299.

Caldji C, Francis D, Sharma S, Plotsky PM, Meaney MJ (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.

Caldji C, Tannenbaum B, Sharma S, Francis DD, Plotsky PM, Meaney MJ (1998). Maternal care during infancy regulates the development of neural systems mediating the expression of behavioral fearfulness in adulthood in the rat. *Proc Nat Acad Sci USA* **95**: 5335–5340.

Champagne F, Francis DD, Mar A, Meaney MJ (in press). Naturally-occurring variations in maternal care in the rat as a mediating influence for the effects of environment on the development of individual differences in stress reactivity. *Physiol Behav*.

Da Cunha C, Roozendaal B, Vazdarjanova A, McGaugh JL (1999). Microinfusions of flumazenil into the basolateral but not the central nucleus of the amygdala enhance memory consolidation in rats. *Neurobiol Learn Mem* **72**: 1–7.

Dickinson-Anson H, McGaugh JL (1997). Bicuculline administered into the amygdala after training blocks benzodiazepine-induced amnesia. *Brain Res* **752**: 197–202.

Francis DD, Diorio J, Liu D, Meaney MJ (1999). Nongenomic transmission across generations in maternal behavior and stress responses in the rat. *Science* **286**: 1155–1158.

Furmark T, Fischer H, Wik G, Larsson M, Fredrikson M (1997). The amygdala and individual differences in human fear conditioning. *Neuroreport* **22**: 3957–3960.

Glue P, Wilson S, Coupland N, Ball D, Nutt D (1995). The relationship between benzodiazepine receptor sensitivity and neuroticism. *J Anxiety Dis* **9**: 33–45.

Gonzalez LE, Andrews N, File SE (1996). 5-HT1A and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. *Brain Res* **732**: 145–153.

Gorman JM, Kent JM, Sullivan GM, Coplan JD (2000). Neuroanatomical hypothesis of panic disorder, revised. *Am J Psychiatry* **157**: 493–505.

Griebel G, Perrault G, Letang V, Granger P, Avenet P, Schoemaker H *et al* (2000). New evidence that the pharmacological effects of benzodiazepine receptor ligands can be associated with activities at different BZ (omega) receptor subtypes. *Psychopharmacology (Berl)* **146**: 205–213.

Gunther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F *et al* (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **92**: 7749–7753.

Hadingham KL, Wingrove P, Le Bourdelles B, Palmer KJ, Ragan CI, Whitiing PJ (1993). Cloning of cDNA sequences encoding human α2 and α3 gama-aminobutyric acidA receptor sub-units and characterization of the benzodiazepine pharmacology of recombinant α1-, α2-, α3- and α5-containing human g-aminobutyric acidA receptors. *Mol Pharmacol* **3**: 970–975.

Haefely W (1994). In: Mohler H, DaPrada M (eds) *The Challenge of Neuropharmacology*. Roche, Basel: Switzerland. pp 15–40.

Helmstetter FJ, Bellgowan PS (1994). Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. *Behav Neurosci* **108**: 1005–1009.

Hodges H, Green S, Glenn B (1987). Evidence that the amygdala is involved in benzodiazepine and serotonergic effects on punished responding, but not discrimination. *Psychopharmacology* **92**: 491–504.

Im WB, Blakeman DP (1991). Correlation between gamma-aminobutyric acidA receptor ligand-induced changes in t-butylbicyclophosphoro[^{35}S]thionate binding and 36Cl$^-$ uptake in rat cerebrocortical membranes. *Mol Pharmacol* **39**: 394–398.

Khan ZU, Gutierrez A, De Blas AL (1996). The α1 and α6 sub-units can co-exist in the same cerebellar GABAA receptor maintaining their individual benzodiazepine-binding specificities. *J Neurochem* **66**: 685–691.

LaBar KS, Gatenby JC, Gore JC, LeDoux JE, Phelps EA (1998). Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. *Neuron* **20**: 937–945.

LaBar KS, LeDoux JE, Spencer DD, Phelps EA (1995). Impaired fear conditioning following unilateral temporal lobectomy in humans. *J Neurosci* **15**: 6846–6855.

Liu D, Tannenbaum B, Caldji C, Francis DD, Freedman A, Sharma S *et al* (1997). Maternal care, hippocampal glucocorticoid receptor gene expression and hypothalamic–pituitary–adrenal responses to stress. *Science* **277**: 1659–1662.

Ma W, Barker JL (1995). Complementary expressions of transcripts encoding GAD67 and GABAA receptor a4, β1 and g1 subunits in the proliferative zone of the embryonic rat central nervous system. *J Neurosci* **15**: 2547–2560.

Maccari S, Piazza PV, Kabbaj M, Barbazanges A, Simon H, LeMoal M. (1995). Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. *J Neurosci* **15**: 110–116.

Maksay G (1993). Partial and full agonists/inverse agonists affect [35S]TBPS binding at different occupancies of central benzodiazepine receptors. *Eur J Pharmacol* **246**: 255–260.

Malizia AL, Cunningham VJ, Bell CJ, Liddle PF, Jones T, Nutt DJ (1998). Decreased brain GABA(A)-benzodiazepine receptor binding in panic disorder: preliminary results from a quantitative PET study. *Arch Gen Psychiatry* **55**: 715–720.

McCarty R, Lee JH (1996). Maternal influences on adult blood pressure of SHRs: a single pup cross-fostering study. *Physiol Behav* **59**: 71–75.

McKernan RM, Quirk K, Prince R, Cox PA, Gillard NP, Ragan CI *et al* (1991). GABAA receptor subtypes immunopurified from rat brain with alpha subunit-specific antibodies have unique pharmacological properties. *Neuron* **4**: 667–676.

McKernan RM, Whiting PJ (1997). Which GABAA-receptor subtypes really occur in the brain? *Trends Neurosci* **19**: 139–143.

Mehta AK, Ticku MK (1999). An update on GABAA receptors. *Brain Res Rev* **29**: 196–217.

Melo de Paula AJ (1977). A comparative study of lormetazepam and flurazepam in the treatment of insomnia. *Clin Ther* **64**: 500–508.

Muller J, Corodimas KP, Fridel Z, LeDoux JE (1997). Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit

conditioned stimulus and to contextual stimuli. *Behav Neurosci* **111**: 683–691.

Myers MM, Brunelli SA, Squire JM, Shindeldecker RD, Hoffer MA (1989). Maternal behavior of SHR rats and its relationship to offspring blood pressures. *Dev Psychobiol* **22**: 29–53.

Oblowitz H, Robins AH (1983). The effect of clobazam and lorazepam on the psychomotor performance of anxious patients. *Br J Clin Pharmacol* **16**: 95–99.

Owens MJ, Vargas MA, Knight DL, Nemeroff CB (1991). The effects of alprazoplam on corticotropin-releasing factor neurons in the rat brain: acute time course, chronic treatment and abrupt withdrawal. *J Pharmacol Exp Therap* **258**: 349–356.

Paxinos G, Watson D (1982). *The Rat Brain in Stereotaxic Coordinates*. Academic Press: New York, NY.

Pesold C, Treit D (1995). The central and basolateral amygdala differentially mediate the anxiolytic effects of benzodiazepines. *Brain Res* **671**: 213–221.

Pitkanen A, Savander V, LeDoux JE (1997). Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. *Trends Neurosci* **20**: 517–523.

Poulter MO, Barke JL, O'Carroll A-M, Lolait SJ, Mahan LC (1992). Differential and transient expression of GABAA receptor a-subunit mRNAs in the developing rat CNS. *J Neurosci* **12**: 2888–2900.

Pritchett DB, Seeburg PH (1991). Type I and type II GABAA-benzodiazepine receptors produced in transfected cells. *Science* **245**: 1389–1391.

Pruessner J, Champagne FA, Dagher A, Meaney MJ (2000). Parental care and stress-induced dopamine release assessed in a PET study of raclopride in young, health human subjects. *Soc Neurosci Abst*.

Ressnick HS, Kilpatrick DG, Dansky BS, Saunders BE, Best CL (1993). Prevalence of civilian trauma and post-traumatic stress disorders in a representative national sample. *J Clin Consul Psychol* **61**: 984–991.

Roy-Byrne P, Cowley DS, Greenblatt DJ, Shader RI, Hommer D (1990). Reduced benzodiazepine sensitivity in panic disorder. *Arch Gen Psychiatry* **47**: 534–538.

Roy-Byrne P, Wingerson DK, Radant A, Greenblatt DJ, Cowley DS (1996). Reduced benzodiazepine sensitivity in patients with panic disorder: comparison with patients with obsessive–compulsive disorder and normal subjects. *Am J Psychiatry* **153**: 1444–1449.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM *et al* (1999). Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature* **401**: 796–800.

Schafe GE, Nader K, Blair HT, LeDoux JE (2001). Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends Neurosciences* **24**: 540–546.

Sieghart W (1995). Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* **47**: 181–234.

Skelton KH, Nemeroff CB, Knight DL, Owens MJ (2000). Chronic administration of the triazolobenzodiazepine alprazolam produces opposite effects on corticotropin-releasing factor and urocortin neuronal systems. *J Neurosci* **20**: 1240–1248.

Stein MB, Walker JR, Anderson G, Hazen AL, Ross CA, Eldridge G *et al* (1996). Childhood physical and sexual abuse in patients with anxiety disorders and in a community sample. *Am J Psychiatry* **153**: 257–275.

Tomaz C, Dickinson-Anson H, McGaugh JL, Souza-Silva MA, Viana MB, Graeff FG (1993). Localization in the amygdala of the amnestic action of diazepam on emotional memory. *Behav Brain Res* **58**: 99–105.

Tweed JL, Schoenbach VJ, George LK, Blazer DG (1989). The effects of childhood parental death and divorce on six-month history of anxiety disorders. *Br J Psychiatry* **154**: 823–828.

Udwin O, Boyle S, Yule W, Bolton D, O'Ryan O (2000). Risk factors for long-term psychological effects of a disaster experienced in adolescence: predictors of post-traumatic stress disorder. *J Child Psychol Psychiatry* **41**: 969–979.

Valentino RJ, Curtis AL, Page ME, Pavcovich LA, Florin-Lechner SM (1998). Activation of the locus cereuleus brain noradrenergic system during stress: circuitry, consequences, and regulation. *Adv Pharmacol* **42**: 781–784.

Wilcox AS, Warrington JA, Gardiner K, Berger R, Whiting P, Altherr MR *et al* (1992). Human chromosomal localization of genes encoding the gamma 1 and gamma 2 subunits of the gamma-aminobutyric acid receptor indicates that members of this gene family are often clustered in the genome. *Proc Natl Acad Sci USA* **89**: 5857–5861.

Wingrove PB, Thompson SA, Wafford KA, Whiting PJ (1997). Key amino acids in the gamma subunit of the gamma-aminobutyric acidA receptor that determine ligand binding and modulation at the benzodiazepine site. *Mol Pharmacol* **52**: 874–881.

Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992). The distribution of 13 GABAA receptor submit mRNAs in the rat brain. **1**: Telencephalon diencephalon, mesencephalon. *J Neurosic* **12**: 1040–1062.

Yang W, Drewe JA, Lan NC (1995). Cloning and characterization of the human GABAA receptor alpha 4 subunit: identification of a unique diazepam-insensitive binding site. *Eur J Pharmacol* **291**: 319–325.

Ymer S, Schofield PR, Draguhn A, Werner P, Kohler M, Seeburg PH (1989). GABAA receptor β submit heterogeneity: functional expression of cloned cDNAs. *EMBO J* **3**: 1665–1671.