Distinct Functional Characteristics of the Lateral/Basolateral Amygdala GABAergic System in C57BL/6J and DBA/2J Mice

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ABSTRACT
It is generally understood that genetic mechanisms contribute to pathological anxiety and that C57BL/6 (B6) and DBA/2J (D2) mice, inbred strains differing markedly in their anxiety-like behaviors, may represent a model system to study these contributions. Because lateral/basolateral amygdala (BLA) GABA<sub>A</sub> receptors help regulate anxiety-like behaviors, we have tested the hypothesis that differences in receptor function/expression may be related to strain-specific differences in experimentally measured anxiety. First, we demonstrated that anxiety-like behaviors in two separate assays were more substantial in D2 mice. Then, using whole-cell electrophysiology of isolated neurons, we found that D2 BLA neurons expressed significantly greater GABA-gated responses than B6 BLA neurons. This was specific for GABA<sub>A</sub> receptors, because N-methyl-D-aspartate-gated responses were similar between strains. At the molecular level, this increased GABA<sub>A</sub> function was associated with higher levels of α<sub>2</sub> subunit mRNA expression in D2 BLA. Finally, to understand the ramifications of these functional and molecular biological differences, we examined both electrically evoked GABAergic responses and spontaneous synaptic currents using whole-cell recordings with in vitro slice preparations. Presynaptic GABAergic function was more robust in D2 compared with B6 slices. Together, our findings suggest that genetic mechanisms differentially represented in these two inbred mouse strains lead to robust differences in pre- and postsynaptic aspects of amygdala GABAergic function.

It is generally recognized that genetic mechanisms make important contributions to neuropsychological disorders. For example, pathological anxiety disorders, such as agoraphobia (Davids et al., 2002) and panic disorder (Merikangas et al., 1998), have familial linkage suggestive of substantial genetic contributions. Experiments with nonhuman primates (BeThea et al., 2004) and rodent models (Trullas and Skolnick, 1993) have largely supported this gene-anxiety relationship. However, the relevant neurobiological mechanisms controlled by these heritable factors remain to be defined. Inbred rodent strains represent an important model in this regard given that individuals within a strain are genetically homogeneous. Strain-by-strain behavioral comparisons and subsequent interstrain breeding, along with extensive genetic mapping, have led to some understanding of the chromosomal loci associated with behavioral responsiveness to anxiolytic drugs (Griebel et al., 2000). More importantly, several anxiety-related "quantitative trait loci" (QTL) or segments of chromosomes that segregate with a particular behavioral phenotype have been identified using inbred and congenic inbred mouse strains (Gill and Boyle, 2005). One of these QTLs is tightly linked to the GABA<sub>A</sub> α<sub>2</sub>/β<sub>3</sub>/γ<sub>1</sub> subunit cluster located on chromosome 5. This may suggest that genetic determinants related to the GABAergic system make important contributions to anxiety-related behaviors. In support of this, investigators using the C57BL/6 (B6), DBA/2J (D2), and related BXD recombinant inbred mouse strains have identified coding-sequence variations in a number of specific genes within QTLs associated with differential behavioral responses to ethanol exposure, some of which encode GABA<sub>A</sub> receptor subunits (Hood and Buck, 2000). Thus, behavior-by-genotype approaches have yielded some understanding of the chromosomal contributions to anxiety-like responses and of specific genetic contributions to other behavioral phenotypes. However, the inclusion of functional

ABBREVIATIONS: QTL, quantitative trait loci; BLA, lateral/basolateral amygdala; B6, C57BL/6 mouse strain; D2, DBA/2J mouse strain; IPSC, inhibitory postsynaptic current; mIPSC, miniature IPSC; QX314-Cl, lidocaine; NMDA, N-methyl-D-aspartate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; TTX, tetrodotoxin.
neurobiological measures in a genotype-by-phenotype experimental approach might provide unique insight into the genetic mechanisms regulating strain-dependent behavioral differences, particularly as they relate to anxiety-associated behaviors.

As with many complex behaviors, numerous brain regions contribute to the expression of anxiety-like behavior. In particular, the amygdala, a limbic forebrain area, seems to provide pivotal regulation of this behavior. Chemical lesions of the amygdala reduce innate anxiety in humans (Masaoka et al., 2003) and nonhuman primates (Amaral, 2002). However, several amygdala subdivisions work in concert to establish/express anxiety-like behaviors. In particular, rodent studies have demonstrated that the lateral/basolateral amygdaloid subdivisions (BLA), the primary site for cortical and thalamic inputs into the amygdala, play a central role in establishing amygdala-dependent anxiety/fear responses. The BLA is critical for the acquisition of learned-fear responses (Killcross et al., 1997) as well as innate expression of anxiety-like behaviors (Sajdyk and Shekhar, 1997). Despite substantial evidence suggesting that anxiety-like behaviors are genetically linked, it is not clear to what extent inherited factors contribute to these BLA-dependent responses.

The neurophysiological mechanisms governing amygdala-dependent anxiety-like behaviors seem to rely upon a balance between excitatory, glutamatergic, and inhibitory GABAergic function. For example, direct activation of lateral/basolateral amygdala GABA_A receptors reduces expression of innate anxiety-like responses (Bueno et al., 2005) and prevents the acquisition of learned fears (Wilensky et al., 1999). Thus, in the context of the current application, BLA GABAergic mechanisms represent a central regulatory component in the expression of fear/anxiety. Whereas recent work suggests that genetic manipulations can alter amygdala-dependent expression of these behaviors that is coincident with altered BLA neurotransmission (Wei et al., 2002), the extent to which genetic mechanisms help to govern BLA GABAergic neurophysiology and ultimately the expression of anxiety-like behavior is entirely unexplored.

Because of the critical role of BLA GABAergic neurophysiology in the regulation of anxiety-like behaviors as well as the contribution of heritable factors to fear and/or anxiety, the current work tests the hypothesis that differences in lateral/basolateral amygdaloid GABA_A receptors are related to genetically driven differences in anxiety-like behavior. To test this hypothesis, we have used behavioral, molecular, and electrophysiological techniques in a comprehensive study of neuronal GABA_A receptor function in two inbred mouse strains. We examined GABAergic function/expression specifically in the B6 and D2 inbred mouse strains because of their distinct anxiety-related phenotypes (Parmigiani et al., 1999; Griebel et al., 2000) and because recombinant inbred strains derived from these strains have been used to identify specific GABA_A subunit genes related to other behavioral phenotypes (Hood and Buck, 2000). Our results may eventually provide insights into the genetic determinants contributing to the physiological mechanisms governing anxiety-like behaviors.

Materials and Methods

**Behavioral Assays.** Four to 5-week-old male B6 and D2 were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed under standard conditions for at least 1 week prior to use. At the time of experimentation, animal ages ranged from 6 to 8 weeks. Prior to making the behavioral measures, pairs of mice in their home cage were placed in the experimental room and exposed to 65-dB “white” noise for 30 min prior to placement in the test apparatus. To avoid order effects and interactions among different behavioral assays, individual mice were tested in a single apparatus.

To measure anxiety with the light/dark box, individuals were placed in the “light” side of a 25 × 25-cm Plexiglas arena divided equally into light and “dark” sides by an opaque Plexiglas insert (Mouse Truscan Activity Arena; Coulbourn Instruments, Allentown, PA). The geometric center of the animal ± 0.8 cm was followed for 300 s by two infrared sensor rings surrounding the entire apparatus, one in the floor plane and one located −5 cm above the floor to measure rearing behavior. Data were collected at 0.25 Hz and analyzed for locomotor activity, time spent in the light and dark compartments, number of light-dark transitions, and number of vertical beam breaks as described previously (McCool et al., 2003). The illumination of the light side was from ambient room lighting and was −650 lumens. Between animals, the apparatus was cleaned with warm water, 70% ethanol, and then warm water and thoroughly dried. All variables are reported as the mean ± S.E.M.

To measure anxiety in an open field (Holmes et al., 2002), animals were placed in the center of 40 × 40-cm Plexiglas arenas (Rat Truscan Activity Arena; Coulbourn Instruments) illuminated by ambient room lighting to −350 lumens at the center of the arena. The geometric center of the animal was followed at a sampling frequency of 1 Hz for 30 min using an infrared sensor ring with a spatial resolution of ±1.3 cm. Data were analyzed in 1- to 5-min bins for total locomotor activity as well as activity around the “margin” and “center” of the arena. Between animals, the apparatus was cleaned with warm water, 70% ethanol, and then warm water and thoroughly dried. All variables are reported as the mean ± S.E.M. and compared using the standard Student’s t test.

**Preparation of Brain Tissue.** Animals were anesthetized with isoflurane and euthanized by decapitation according to the Institutional Animal Care and Use Committee-approved protocol that is consistent with the Public Health Service policy on the humane care and use of laboratory animals. In some cases, animals used in the behavioral experiments were also used for tissue preparation. Exposure to the behavioral tests did not alter any of the dependent variables, and data from these animals were pooled with apparatus-naive, age-matched, chow-fed controls. Coronal brain slices (300 μm) were prepared as described previously (Floyd et al., 2003). For in vitro slice preparations, 100 μM ketamine was added to the modified Ringer’s solution during preparation to minimize excitotoxicity. Slices were stored in standard oxygenated Ringer’s at room temperature for at least 1 h and up to 8 h following preparation. For some studies, individual neurons were isolated from coronal brain slices by incubation of the tissue with 0.5 to 0.7 mg/ml Pronase (EMD Biosciences, San Diego, CA) in oxygenated Ringer’s solution as described previously (Floyd et al., 2003). These isolated neurons were plated onto glass coverslips coated with 0.2% alginic acid, allowed to settle for several minutes, and rinsed with a HEPES-buffered saline. Sliced tissue used for slice patch-clamp electrophysiology was allowed to incubate in a drug-free oxygenated Ringer’s solution at 25°C for 1 h before recording.

**Electrophysiology on Acutely Isolated Neurons.** Patch-clamp recordings were performed in the whole-cell mode on isolated amygdala neurons using established procedures as described previously (Floyd et al., 2003). To maintain consistency between our previous findings and the current study, the intracellular pipette solution contained 100 mM CsCl, 10 mM HEPES, 10 mM EGTA, and 4 mM Mg-ATP, with pH adjusted to 7.2 with CsOH (osmolality adjusted to 300–305 mmol/kg with sucrose). Cells were continuously perfused with HEPES-buffered saline (Floyd et al., 2004) containing 0.2 μM tetrodotoxin. Drugs were applied to these isolated cells from a stepper-motor driven array of glass tubes (0.7-mm diameter).
placed within 50 μm of each cell. All data were obtained from drug-on rates of <80 ms. Current responses to applied drugs were acquired with an AxoPatch 200B amplifier and pClamp 9.0 (Molecular Devices/Axon Instruments, Union City, CA), digitized at 10 kHz, filtered at 2 kHz, and stored for later analysis. Current densities were calculated by dividing current amplitude by apparent cell size (e.g., whole-cell capacitance in picoFarads); this latter measure was obtained prior to drug application on each cell from exponential fits of the membrane current-response to square-wave depolarizations. Access resistance was 12.9 ± 1.1 MΩ for B6 neurons (n = 35) and 12.7 ± 1.0 MΩ in D2 neurons (n = 28; P < 0.05, t test). Whole-cell capacitance in the same dataset was significantly different among different strains and is presented in the text. Cells with a whole-cell capacitance <10 pF were presumed to be GABAergic interneurons (McDonald, 1985) and were excluded from the study. Cells with “leak” currents greater than 100 pA at a holding potential of 30 to -40 mV were discarded. Drugs were applied to the cell via an array of glass capillary tubes mounted onto a computer-controlled stepper motor and placed within 200 μm of the cell.

**Slice Recordings.** Slices were continuously perfused with oxygenated Ringer’s solution containing 20 μM 6,7-dinitroquinoxaline-2,3-dione and 50 μM di-2-aminophosphonovinic acid to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate and N-methyl-d-aspartate (NMDA) receptors, respectively. All drugs were applied directly to the Ringer’s solution via calibrated syringe pumps. Methods for “blind” whole-cell recordings from “adult” neurons within slices were similar to those reported previously (Weiner et al., 1997). For measuring electrically evoked responses, patch electrodes were filled with an intracellular pipette solution containing 130 mM potassium gluconate, 13 mM KCl, 10 mM HEPES, 1.1 mM EGTA, 0.1 mM CaCl₂, 2 mM Mg-ATP, and 0.3 mM Na-GTP, with pH adjusted to 7.2 with KOH (osmolality adjusted to 290–300 mmol/kg with sucrose), and neurons were voltage-clamped at -40 to -35 mV (to increase the chloride electrochemical driving force). This solution contains a physiologically relevant concentration of chloride. Inhibitory postsynaptic currents (IPSCs) were elicited every 20 s by brief (0.2 ms) electrical stimulation within the BLA using platinum/iridium concentric bipolar-stimulating electrodes with an inner pole diameter of 25 μm and a resistance of 75 MΩ (FHC Inc., Bowdoinham, ME). Paired-pulse ratios were calculated as the second IPSC amplitude divided by the amplitude of the first IPSC, and these data are expressed as mean ± S.E.M. Variables were compared between strains using standard Student’s t test or two-way ANOVA depending upon the experimental design.

To record spontaneous GABAergic synaptic events, we attempted to use a CsCl-based intracellular solution similar to the isolated cell studies but found that it was not technically feasible in BLA slices from adult mice. Therefore, we used a recently reported method where neurons are depolarized to increase chloride driving force (Liang et al., 2006). The intracellular potassium gluconate/KCl described in the preceding paragraph was replaced with 130 mM cesium gluconate and 2 mM QX314-Cl (lidocaine) to reduce neuron excitability at these depolarized potentials. As with the original intracellular solution, osmolality was adjusted to 290–300 mmol/kg using sucrose. Tetrodotoxin (TTX; 1 μM) was delivered to the slice to isolate tetrodotoxin-insensitive “miniature” IPSCs from spontaneous IPSCs. In a separate study, this concentration of TTX blocked 98% of electrically evoked IPSC amplitude (data not shown). BLA neurons were held at 0 mV to provide a sufficient driving force to enhance event rise time. Analysis was performed using Mini-Analy- 6.0.3 (Synaptosoft Inc., Decatur, GA) with threshold criteria of 5 pA amplitude and 30 pA · ms area. “Frequency” was calculated from the interevent interval, whereas “amplitude” represented the absolute difference between the estimated baseline and maximal current of each event. Amplitude and frequency data were evaluated as cumulative distributions and statistically assessed using the Kolmogorov-Smirnov (K–S) test.

All recordings were acquired with an Axoclamp 2B amplifier, digitized with a Digidata 1200B, and analyzed offline using pClamp 8.0/9.2 software (Molecular Devices/Axon Instruments, Foster City, CA). Whole-cell access resistances were in the range of 10 to 20 MΩ, and access resistance was monitored by measuring the size of the capacitative transient in response to a -5 mV step command. Experiments were abandoned if access resistance changed by >20% during the recordings. At least 5 min was allowed for equilibration of the pipette solution with the intracellular milieu before commencing recordings.

**Preparation of RNA and Real-Time Reverse Transcription-PCR.** BLA was dissected from coronal slices after tissue preparation, frozen immediately on dry ice, and stored at -80°C. Total RNA from individual animals was prepared from this fresh-frozen lateral/basolateral tissue (10–15 mg) as described previously (Floyd et al., 2003). As a standard, total forebrain RNA was prepared from a tissue mixture containing a single B6 brain and single D2 brain using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA).

Reverse transcription and real-time PCR were performed as described previously (Floyd et al., 2003). Gene-specific PCR primers and 5'-6FAM/3’-BHQ1 doubly labeled oligonucleotide probes (Integrated DNA Technologies, Coralville, IA) were used with the 5’- exonuclease assay for real-time PCR. Fluorescence was monitored on an Opticon 2 DNA Engine (MJ Research, Waltham, MA). Relative expression levels were derived from C(T) values for each subunit that were standardized to serial dilutions of total forebrain RNA (e.g., relative standard curve method) derived from an equal mixture of both B6 and D2 tissue. PCR primers and TaqMan probes were designed from murine GABA(A) subunit cDNA sequences available from GenBank using PrimerExpress software (Applied Biosystems, Foster City, CA). When designing primers/probes for the various GABA(A) α subunits, regions with >80% identity/20 bp were excluded from consideration. The resulting primers/probes were deemed gene-specific if they did not detect genes other than the intended target using standard BLASTN searches of GenBank. The real-time primer/probe sets are given in Table 1. The GABA(A) γ2 primer/probe set does not distinguish between the L and S splice variants of this subunit and lies 3’ of the stop codon, significantly downstream from the 5’-coding sequence polymorphism previously identified in the B6, D2, and BXD mouse strains (Hood and Buck, 2000).

**Results.**

**Comparison of Anxiety Behavior in B6 and D2 Mice.** The “innate” anxiety of B6 and D2 mice was tested in two different behavioral apparatus. During 300 s in the light/dark box, B6 mice (n = 12) spent significantly more time than D2 mice (n = 10) in the light side of the apparatus (153.8 ± 14.6 versus 86.4 ± 10.4 s, P < 0.01, t test; Fig. 1A) and were quicker to re-enter the light side following their initial egress (re-entry latency; 23.7 ± 3.8 versus 42.0 ± 8.5 s, P < 0.05, t test). The strain-specific differences in these “anxiety”-related variables are similar to those reported previously (Coscullula et al., 1987) and contrast with strain-similar measures of locomotor activity (Fig. 1B). The total amount of move time (212.1 ± 11.9 s in B6 versus 207.0 ± 3.4 s in D2), the total number of moves (141.9 ± 15.9 in B6 versus 146.2 ± 12.1 in D2), the number of transitions between the light and dark compartments (10.8 ± 1.3 in B6 versus 14.4 ± 1.3 in D2), and the total distance moved (1.32 ± 0.07 m in B6 versus 1.58 ± 0.14 m in D2) were not significantly different between strains.

It is possible that the apparent differences in innate anxiety in the light/dark box represent immediate responses to this particular novel environment that are not representative of “resting” anxiety levels in these strains. To test this, we
Anxiety-like behavior measured in an open field and in the light/dark box is greater in D2 mice. A, the time D2 mice spent in the light side of the light/dark box (Time in Light) was significantly less (\(t\) test, \(n = 10\)) compared with B6 mice (\(n = 12\)). D2 mice also took longer to re-enter the light side following their initial egress into the dark compartment (Re-entry Latency; \(t\) test). D2 mice may also leave the light side sooner following their initial placement (Egress Latency; \(t\) test).

B6 and D2 mice were similar across several different locomotion-dependent variables in the light/dark box including Movement Time (in seconds), the total Number of Moves, and the number of Light/Dark Transitions. C and D, in the open field, D2 mice (\(n = 6\) for each strain) spent less time (C) and traveled less distance (D) in the “center” of the open field. To demonstrate that these were stable behaviors, measures were binned in 5-min intervals across the entire 30-min assay and plotted as shown. Two-way ANOVA across time bin and mouse strain indicated significant main effect of strain but not time bin for both variables (see Results) and Bonferroni’s post-tests indicated significant differences between strains at most of the time bins (\(*, P < 0.05; **, P < 0.01; ***, P < 0.001\)).

**Table 1.** Real-time PCR primer/probe sets derived from mouse subunit cDNAs

| Gene (Genbank No.) | nt<sup>a</sup> | Primer<sup>c</sup> | Probe
|-------------------|-------------|-----------------|-----|
| GABA<sub>α</sub> 1 (M63436) | 1811–1895 | CCCATCAATAGTTCCCTTTTAGTCGTATAGGA | TCTAGCTCTCCACTACGAGAATTCTTT
| GABA<sub>α</sub> 2 (M86567) | 1157–1232 | ATTACGTGCCGAGGACCACCTTTTCTTCTGCCTGCGC | AAGATCTTTCTTCTTACACACCTTCCAAAAGTG
| GABA<sub>α</sub> 3 (M86568) | 1408–1475 | CGAGTCTCCACCTCCACTGCTCTCTCAMTGGAGATTTGAAGAAGCCACAACATCGGCTGAGACACTTT | TCTCTTGCTCCACACTGGAATGTA
| GABA<sub>α</sub> 4 (XM_132088) | 572–639 | TGGCTGGCGCGCTTTTTTTTTCTCGGACACATT | AAGCAGATCCCCAGGACAGAACACTCAAG
| GABA<sub>β</sub> 1 (U14418) | 166–232 | GAGGAAACAGCCACAACTGACGCTGGACACCT | TCTCTTGCTCCACACTGGAATGTA
| GABA<sub>β</sub> 2 (U14419) | 41–76 | TGGATCTCTTCTCCACTGGAATCAGCAGCCTT | CTGAAATGCTCCACACTGGAATGTA
| GABA<sub>β</sub> 3 (U14420) | 1226–1297 | TGGACGCAGCTTTGTCTTCTTACTGAACTTCCCTT | AACTGACAGAATCCAGAATCTATCTTCTT
| GABA<sub>γ</sub> 2 (NM_008073) | 1922–1990 | CAGTCCAAATCTGGATATCTTTCCTGTGCAAG | TTTGATTTTCTCAGCTGACCTTT
| GAPDH<sup>d</sup> (M32599) | 757–833 | TGTGCCCTCCTTGATCTGAGATCCTCCTTCCACACT | CCCCGCGAGAGAGACTGGACAGTATG

<sup>a</sup> nt, nucleotide.
<sup>b</sup> cDNA region encompassed by the PCR product.
<sup>c</sup> Sequences are shown 5' to 3'.
<sup>d</sup> Upstream primer top, downstream primer bottom.
<sup>e</sup> Glyceraldehyde phosphate dehydrogenase.
also measured anxiety-like behavior in these strains of mice in the open field but over a 30-min period (Holmes et al., 2002), reasoning that more long-term responses to novel stimuli may better reflect “trait” differences between strains. Overall, B6 mice (n = 6 in each strain) spent significantly more time in the center of the open field (10.3 ± 0.4 versus 3.4 ± 0.4 min in D2, P < 0.001, t test), traveled more distance in the center (1.92 ± 0.14 versus 0.74 ± 0.09 m in D2, P < 0.001, t test), and entered the field center more frequently (109.8 ± 7.5 versus 60.7 ± 7.4 entries in D2, P < 0.001, t test). When these anxiety-related variables were binned in 5-min intervals and compared across strains, there was a significant main effect of strain for center time (Fig. 1C; F = 204.8, P < 0.0001, two-way ANOVA), for center distance (Fig. 1D; F = 112.2, P < 0.0001, two-way ANOVA), and for center entries (data not shown; F = 59.7, P < 0.0001, two-way ANOVA) but not of time bin. This suggests that the behavioral differences between B6 and D2 mice in the open field were stable across the entire 30-min assay. Indeed, this is supported by pairwise comparisons of each time bin using Bonferroni’s post-test that indicated significant differences between strains for most of the 5-min bins for both center time and center distance (Fig. 1, C and D) and for both the first and last bin in the center entries data (data not shown). In contrast, the total number of moves (260.2 ± 5.8 versus 272.0 ± 3.9) and total distance moved (7.12 ± 0.35 versus 7.20 ± 0.28 m) during the entire 30-min test period and across individual 5-min time bins was not significantly different between strains. However, B6 mice spent significantly more time moving than D2 mice during the test (22.8 ± 0.5 versus 20.6 ± 0.2 min, P < 0.01, t test).

**Functional Receptor Levels in Lateral/Basolateral Amygdala.** To understand the neurophysiological basis for these pronounced differences in innate anxiety-like behavior, we examined two amygdala neurotransmitter systems that are known to modulate anxiety behaviors. Using whole-cell patch-clamp recordings of acutely isolated BLA neurons, current responses to GABA (1 μM–3 mM; Fig. 2A) were dose-dependent and similar to previous findings in rat (McCool et al., 2003) and monkey (Floyd et al., 2004). After fitting to a standard logistic equation, GABA potency was not different between strains with the log(EC50) values of −4.56 ± 0.08 for B6 neurons (n = 11; 27 μM) and −4.53 ± 0.14 for D2 neurons (n = 11; 30 μM). However, GABA was significantly more efficacious at the 1 μM, 0.1 mM, 1 mM, and 3 mM concentrations in D2 neurons (Fig. 2B, P < 0.05 at each concentration, two-tailed t test). Absolute current densities at these concentrations were 17.9 ± 3.6 (1 μM), 91.1 ± 10.9 (0.1 mM), 122.4 ± 13.0 (1 mM), and 129.6 ± 15.7 pA/pF (3 mM) in D2 neurons and 75.7 ± 21.2 (1 μM), 57.9 ± 10.0 (0.1 mM), 74.0 ± 12.6 (1 mM), and 81.2 ± 13 pA/pF (3 mM) in neurons from B6 mice. At every concentration, the absolute amplitude of current responses was also larger in D2 neurons compared with B6 neurons. However, these measures were not significantly different between strains due to the substantial cell-to-cell variance of whole-cell current amplitude.

To compare the kinetic properties of B6 and D2 GABA receptors, we used the 3 mM responses from this concentration-response dataset. During the 4 s of drug exposure, currents expressed by neurons from both strains desensitized to a similar extent. Only 19.2 ± 3.4 and 20.7 ± 2.5% of the current remained at the end of the drug application in B6 and D2 neurons, respectively (P > 0.05, t test). Furthermore, the ratio of the peak current amplitude to the current amplitude at the end of the drug application was not significantly different between B6 and D2 neurons (6.8 ± 1.0 in B6 neurons
Unexpectedly, neurons isolated from D2 lateral/basolateral appeared significantly smaller than their B6 counterparts, with whole-cell capacitance being 14.2 ± 0.8 pF for D2 neurons and 19.0 ± 1.9 pF for B6 neurons (P < 0.05, two-tailed t test; Fig. 2C). The apparent cell-size difference between strains suggests that normalizing current amplitude to whole-cell capacitance could bias our findings of strain-specific differences in functional GABA<sub>A</sub> receptors. To test this, we determined the concentration-response data for NMDA in the presence of 3 μM glycine in the acutely isolated neurons (Floyd et al., 2003). Application of NMDA/glycine to isolated cells from both strains resulted in concentration-dependent changes in membrane current (Fig. 3A). However, the concentration-response relationships derived from each strain were similar to each other with respect to both NMDA potency and efficacy (Fig. 3B). log(EC<sub>50</sub>) values, −4.31 ± 0.17 for D2 neurons (n = 5, ~49 μM) and −4.49 ± 0.04 for B6 neurons (n = 7, ~33 μM), were not significantly different (P > 0.05, t test). These potencies are very similar to that reported for rat BLA NMDA receptors (Floyd et al., 2003). More importantly, there was no difference in apparent NMDA current density (picoamperes per picoFarads) across all of the concentrations tested (1 μM–1 mM; P > 0.05 at each concentration). For example, current densities at 1 mM NMDA/3 μM glycine were 28.2 ± 6.9 pA/pF in B6 BLA neurons and 25.4 ± 5.1 pA/pF in D2 neurons (P > 0.05, t test). As with the GABA studies, the whole-cell capacitance of B6 neurons (23.5 ± 1.2 pF; Fig. 3C) was significantly larger than D2 apparent cell size (14.6 ± 1.2 pF; P < 0.01, two-tailed t test) determined in this experiment. Thus, despite the differences in apparent cell size, these findings suggest that the functional densities of somatic NMDA receptors, at least as represented in the isolated cell preparation, are similarly expressed in these mouse strains while GABA<sub>A</sub> receptors are not.

**GABA<sub>A</sub> Receptor Pharmacology.** The preceding data clearly imply that BLA GABA<sub>A</sub> receptors are functionally more robust in isolated neurons from D2 BLA. These isolated cell preparations offer the significant benefit over intact tissue of allowing investigator to test multiple drug concentrations on the same neuron in a relatively short period of time. We took advantage of this to determine whether the apparent strain differences in functional GABA<sub>A</sub> receptors were associated with substantial alterations in receptor subunit contributions. Receptor modulation by benzodiazepine agonist diazepam requires the presence of benzodiazepine-sensitive α subunit (α<sub>1–3,5</sub>) as well as a γ subunit (reviewed in Sieghart, 1994). In our isolated neuron preparation, diazepam (0.3 μM) facilitated current responses to 3 μM GABA. The magnitude of this facilitation depended upon whether neurons were exposed to coapplied GABA/diazepam or were pretreated with diazepam alone for ~30 s prior to application of GABA/diazepam (Fig. 4A). This phenomenon was not explored further. However, regardless of the exposure paradigm, diazepam facilitated GABA responses to a similar ex-
tent in neurons from both strains \((n = 5\) for B6 and \(n = 8\) for D2; Fig. 4A), suggesting that there were no obvious differences related to the contribution by subunits that confer benzodiazepine sensitivity.

There are several reports of subunit-dependent shifts in the sensitivity of \(\text{GABA}_\alpha\) receptors to acute ethanol, especially for extra-synaptic receptors containing the \(\delta\) subunit (Wallner et al., 2003). Therefore, we established complete concentration-response curves for ethanol \((1–100\, \text{mM})\) when applied along with 3 \(\mu\text{M GABA}\) (Fig. 4B). Two-way ANOVA of the cumulative concentration-response data across strains (Fig. 4B) did not reveal any significant main effect of ethanol concentration \((F = 0.11)\) but a main effect of mouse strain \((F = 4.1; P < 0.05)\) and a significant interaction between these variables \((F = 2.9; P < 0.05)\). Bonferroni’s post-tests revealed a significant difference between strains at the highest ethanol concentration tested \((100\, \text{mM}; P < 0.05)\). This modest 10% inhibition of GABA currents by 100 mM ethanol in acutely isolated B6 neurons is similar to previous findings using acutely isolated rat lateral/basolateral amygdala neurons (McCool et al., 2003). These data appear to differ from reports of ethanol facilitation of \(\text{GABA}_\text{ergic}\) synaptic responses in vitro (Roberto et al., 2003; Ariwodola and Weiner, 2004; Carta et al., 2004; Wu et al., 2005), and rat BLA \(\text{GABA}_\text{ergic}\) synaptic currents also appear to be facilitated by acute ethanol (Roberto et al., 2006). These latter findings may suggest a substantial presynaptic contribution to the acute ethanol sensitivity of the BLA \(\text{GABA}_\text{ergic}\) system as well. Regardless, the absence of any ethanol facilitation of the GABA-gated currents measured in isolated cells suggests that there is unlikely to be any substantial differences between strains with regard to ethanol-sensitive, delta subunit-containing receptors. In summary, our pharmacological characterizations suggest that the increased \(\text{GABA}_\alpha\) receptor function in D2 BLA neurons is not associated with substantial shifts in subunit contributions.

**GABA\(_\alpha\) Subunit mRNA Expression in B6 and D2 Mice.** To understand the molecular mechanisms responsible for elevated functional receptors in lateral/basolateral neurons isolated from D2 mice, we examined the lateral/basolateral amygdala mRNA expression profile for various \(\text{GABA}_\alpha\) receptor subunits. Subunit expression in the lateral/basolateral amygdala of individual D2 and B6 mice was assessed by real-time reverse transcription-PCR (see Materials and Methods). Relative levels of an individual subunit in each sample were normalized to the expression of the ubiquitous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for differences in RNA quality between each sample. For the \(\text{GABA}_\alpha\) \(\alpha_2\) subunits, the \(\alpha_2\) and \(\alpha_3\) subunits were relatively enriched in lateral/basolateral amygdala \((\geq1.5\)-fold above total forebrain\), whereas the \(\alpha_1\) and \(\alpha_4\) subunits were 80 to 100% of the levels found in forebrain. Although most \(\alpha\) subunits were not different between strains (Fig. 5A), the \(\alpha_2\) subunit mRNA was significantly more abundant in D2 mice \((3.41 \pm 0.13\) units\) relative to B6 mice \((1.72 \pm 0.11\) units; \(P < 0.0001\), \(t\) test). There was no significant difference between B6 and D2 mice for any of the \(\beta\) subunit mRNAs.
Fig. 5. B6 and D2 lateral/basolateral amygdala express different levels of some GABA<sub>A</sub> receptor subunit mRNAs. A, summary of the relative levels for the most predominant α subunit mRNAs in the lateral/basolateral amygdala (see Floyd et al., 2004). Only α<sub>2</sub> expression was significantly different between B6 (n = 5) and D2 mice (n = 4; *** P < 0.001). B and C, expression of β<sub>1</sub>, β<sub>2</sub> and γ<sub>2</sub> subunits was similar between strains. D, the relative levels of the ubiquitous gene GAPDH were not different between strains. Because GAPDH was used to normalize expression levels of GABA<sub>A</sub> subunit mRNAs in any given sample, these data indicate that the normalization process did not bias our results.

BLA GABAAergic Synaptic Physiology. Because acutely isolated neurons differed significantly in their functional GABA response but showed no notable changes in receptor subunit message, with the exception of the α<sub>2</sub> subunit, it is possible that the two mouse strains possessed differences at a more complex level. To examine this, we examined pharmacologically isolated GABA<sub>A</sub> receptor-mediated IPSCs using whole-cell recordings in an in vitro slice preparation. Under our recording conditions (i.e., potassium gluconate internal with holding potentials of -40 to -35 mV), holding membrane currents in B6 and D2 neurons were 49.4 ± 12.8 pA in B6 neurons and 45.7 ± 17.8 pA in D2 neurons (P > 0.05, t test).

To measure basal differences in evoked synaptic GABA release between the two strains, a paired-pulse ratio was evaluated (Fig. 6A) in BLA neurons using interstimulus intervals of 25, 50, and 250 ms. GABA IPSC paired-pulse ratios from D2 mice were significantly smaller than those recorded from B6 BLA neurons (Fig. 6B) at the 25-ms (D2 = 1.16 ± 0.04, B6 = 1.48 ± 0.07; P < 0.01, t test) and the 50-ms interpulse intervals (D2 = 1.05 ± 0.02, B6 = 1.27 ± 0.08; P < 0.01, t test), but not for the 250-ms interpulse interval (D2 = 0.79 ± 0.04, B6 = 0.96 ± 0.11; P > 0.05, t test). These differences in paired-pulse facilitation might be explained by the slow kinetics of evoked GABAergic responses under our recording conditions. Therefore, we analyzed electrically evoked IPSCs using a stimulus intensity that evoked a near-maximal response (i.e., where response amplitude begins to plateau in relation to stimulus intensity; data not shown). The amplitude of this IPSC from D2 neurons (n = 10) was 311.7 ± 37.4 and 188.9 ± 29.7 pA from B6 BLA neurons (n = 10; P < 0.05, t test). Despite these amplitude differences, the 10 to 90% rise times of the IPSCs were similar between B6 neurons (10.8 ± 1.2 ms) and D2 neurons (9.2 ± 1.3 ms; P > 0.05, t test). The relatively slow-onset kinetics in both strains can be attributed to room temperature recordings and the modest chloride electrochemical potential in our physiologically relevant potassium gluconate internal solution (see Materials and Methods). We also examined the decay kinetics of the evoked response. Two B6 neurons and one D2 neuron had IPSCs that could be fit only by a single exponential and were excluded from further kinetic analysis. The 90 to 10% decay time, 169.5 ± 33.8 ms in B6 neurons and 184.5 ± 35.8 ms in D2 neurons, was not significantly different between strains (P > 0.05, t test). Two-component exponential fits of IPSC decay (Edwards et al., 1990) failed to reveal any differences in either component. Furthermore, the fast component contributed 75.2 ± 4.2% of the IPSC in B6 neurons and 76.6 ± 4.2% in D2 neurons (P > 0.05, t test). Thus, strain-dependent differences in the kinetic parameters of the evoked IPSC are unlikely to have influenced the paired-pulse data.

To more fully understand any pre- or postsynaptic contri-
A major finding of this study is that D2 mice exhibit higher basal levels of anxiety-like behavior in two different assays compared with B6 mice. Taken together, these data suggest that anxiety differences between B6 and D2 are more likely a reflection of stable innate behaviors rather than short-term responses to a novel environment. Yet, D2 mice exhibit greater GABA_A receptor function in the lateral/basolateral amygdala. In isolated cells, this was manifest as a large GABA-gated current density in D2 neurons. This is consistent with the higher density of forebrain flunitrazepam binding in D2 mice compared with their B6 counterparts (Robertson, 1980). Increased relative expression of α_2 subunit mRNA in this strain could contribute to this enhanced functional response. However, it is difficult to interpret increased expression of a single α subunit since functional GABA_A receptors contain at least α and β subunits (Pritchett et al., 1989). The similar benzodiazepine sensitivity of whole-cell GABA currents in B6 and D2 BLA neurons is consistent with similar α/β/γ subunit contributions in both strains as well.

We conclude then that the large GABA_A current density in D2 neurons is not due solely to subunit-specific transcriptional mechanisms. Rather, translational and post-translational mechanisms may also play a significant role in strain-dependent functional differences. However, the higher levels of α_2 subunit mRNA expression in D2 lateral/basolateral amygdala may suggest that D2 neurons could potentially express GABA_A receptors with substantially larger contributions of the α_2 subunit. It should be noted that, with the experimental approach used here, we cannot rule out the possibility that the increased α_2 subunit mRNA levels in D2 tissue may reflect differential expression of this subunit within a specific subclass of neurons. Single-cell PCR approaches could address this concern but are not themselves robustly quantitative and would therefore provide little insight with regard to the increased receptor function in D2 neurons. Alternatively, the α_2-selective modulator zolpidem might yield some insight into the functional contributions of different α subunits; however, our mRNA expression data clearly imply that α_1-containing receptors may not be the predominate isoform in this brain region. Furthermore, recent evidence suggests that BLA α_1-containing and α_2-containing GABA_A receptors are segregated to distinct synaptic compartments (Marowsky et al., 2004). This would further complicate estimations of α_2 contributions by measuring strain-dependent differences with α_2-selective compounds. Once α_2-selective pharmacological agents become available, this can be tested directly.

In addition to increased GABA-gated currents in isolated cells and increased expression of GABA_A α_2 subunit in D2 basolateral amygdala, we found strain-dependent differences in synaptic GABAergic function as well. Paired-pulse facilitation was lower, and the frequency of spontaneous TTX-resistant miniature IPSCs was higher at GABA synapses in D2 BLA neurons. The dramatic differences in mIPSC frequency, 3.7 ± 0.5 Hz in D2 neurons and 1.9 ± 0.4 Hz in B6 neurons, are particularly noteworthy given that they were associated with more modest differences in mIPSC amplitude, 11.8 ± 0.2 pA in D2 neurons and 12.9 ± 0.2 pA in B6 neurons. Because event amplitude was actually smaller at D2 GABAergic synapses, the frequency differences are not...
likely to be related to strain-dependent differences in event detection. Therefore, we conclude that these frequency data together with the paired-pulse data are consistent with greater presynaptic GABA release at D2 BLA synapses. This increased presynaptic function could represent an adaptation to a diminished targeting of GABA<sub>A</sub> receptors to post-synaptic specializations in this strain as suggested by their smaller mIPSC amplitude. This is an attractive hypothesis given that it could lead to an accumulation of extra-synaptic receptors, which in turn might explain the increased whole-cell currents that we observed in isolated neurons from the D2 strain. Conversely, reduced amplitude might also reflect an adaptive response to the increased presynaptic release of GABA per se. Such adaptations could also include altered biophysical characteristics like decreased single channel conductance. Indeed, hippocampal mIPSC amplitude is reduced in GABA<sub>A</sub> α<sub>1</sub> knock-out mice (Goldstein et al., 2002). These findings suggest that distinct subunit contributions at BLA GABAergic synapses might also contribute to the strain-specific mIPSC amplitudes as well.

It is well known that experimental activation of the basolateral amygdala GABAergic system clearly decreases anxiety-like behavior in rodents (Bueno et al., 2005). This led us to hypothesize that D2 mice, with greater levels of anxiety-like behavior, might possess lower GABAergic function. Contrary to this expectation, our studies demonstrate that D2 mice have higher levels of both anxiety-like behavior as well as GABA function compared with B6 mice. These apparently paradoxical findings are not unique to this study. For example, higher levels of benzodiazepine binding are present in D2 mice despite the greater susceptibility of this strain to audiogenic seizures (Robertson, 1980). One possible interpretation is that the endogenous activity of amygdala GABA<sub>A</sub> receptors plays a limited role in the generation of anxiety-like behaviors. However, microinjection of bicuculline and picrotoxin into the BLA increases expression of anxiety-like behavior (Sanders and Shekhar, 1995). This suggests that the BLA GABAergic system is not only actively regulating anxiety-like behavior but could act to either increase or decrease behavioral responsiveness depending upon the salience of a given environment. This could, in turn, imply that the enhanced GABAergic function is the result of heightened over-
all behavioral responsiveness in D2 mice. For example, this increased GABAergic function may act as an adaptive response to additional “pro-anxiety” influences, such as enhanced excitatory synaptic function within the BLA or increased “output” to downstream regions like the central amygdaloid nucleus or prefrontal cortex.

A final point deserving some discussion is our finding that isolated D2 BLA neurons have a smaller whole-cell capacitance than isolated B6 neurons. It is possible that this is a reflection of decreased dendritic arborization or smaller somatic diameter in D2 neurons. However, given that acutely isolated BLA neurons lack significant dendritic processes in general, smaller soma are a more likely explanation. This would imply that D2 BLA neurons are more compact than their B6 counterparts. These differences in apparent cell area may significantly influence neuronal responses to synaptic inputs. For example, treatments that change whole-cell capacitance can alter both action potential amplitude and duration (Gu et al., 2000). Thus, the apparent increased GABAergic preand postsynaptic function in D2 basolateral amygdala neurons may reflect an adaptation to increased neuronal excitability. Regardless, a more complete understanding of the passive membrane properties regulating the excitability of D2 and B6 BLA neurons seems warranted. Alternatively, the increased GABAergic function and smaller somatic size of D2 BLA neurons could be an adaptive response to some as yet uncharacterized imbalance between excitatory and inhibitory influences. In this case, a stronger intrinsic GABAergic influence would be required to maintain balance with extrinsic excitatory inputs. For example, experimentally induced seizures generated by electrical kindling of this brain region seem to directly affect the GABAergic system (Rainnie et al., 1992). Manipulation of amygdala GABAergic function can also alter seizure threshold after this electrical kindling (Schwark and Haluska, 1987). These findings suggest a limited capacity of the intrinsic inhibitory system within the lateral/basolateral amygdala. Strongly anxiogenic stimuli may be able to overwhelm GABAergic function and, as in D2 mice, result in more pronounced anxiety-like behavior.

In conclusion, we have shown that two inbred mouse strains differ significantly in their anxiety-like behavior also have substantial soma-size and neurophysiological differences in a relevant brain region. Whereas their differences in pre- and postsynaptic GABAergic function seemed to contradict those expected based on behavioral outcomes, the distinct neuronal sizes associated with each strain raises the interesting possibility that neuroanatomical differences between these inbred strains may dictate many of their behavioral and neurophysiological differences. Although morphometric and neuroanatomical comparisons of amygdala between different inbred strains have not been performed, cell number in any given brain region seems to have a significant genetic influence (Seecharan et al., 2003). It is reasonable to suggest then that anatomical differences and variations in neurotransmitter receptor expression/function both may play a significant role in regulating anxiety-like behaviors.

References


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