Chronic Ethanol Ingestion Facilitates N-methyl-D-aspartate Receptor Function and Expression in Rat Lateral/Basolateral Amygdala Neurons

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Abstract

Withdrawal anxiety following chronic alcohol is likely to contribute to drug-seeking and relapse in alcoholics. The brain regions regulating fear/anxiety behaviors, especially neurotransmitter systems with acute ethanol sensitivity, are potential targets for chronic ethanol-induced adaptations. We have therefore examined N-methyl-D-aspartate (NMDA) receptors following chronic ethanol ingestion in rat lateral/basolateral amygdala. Whole cell patch clamp measurements indicate that chronic ethanol ingestion significantly increased NMDA receptor current density. This enhanced NMDA receptor function was also associated with an increase in ifenprodil inhibition and a decrease in apparent calcium-dependent current inactivation. These findings suggest that NR2B-containing receptors may be specifically enhanced and suggest that processes dependent upon calcium influx through amygdala NMDA receptors may potentially be enhanced by chronic ethanol ingestion. We measured subunit mRNA expression to investigate possible molecular mechanisms that control functional receptor adaptations to chronic ethanol. Quantitative real-time RT-PCR demonstrated that NR1 subunit mRNA expression, but not NR2 or NR3 expression, was enhanced in samples from chronic ethanol-exposed animals. Single-cell RT-PCR was then used to confirm that NR2 mRNA expression was unaltered by chronic ethanol. Most GAD-1, presumed projection neurons expressed both NR2A and NR2B mRNAs; and this profile did not change during chronic ethanol exposure. Our results suggest that both transcriptional and non-transcriptional adaptations to chronic ethanol ultimately contribute to alterations in NMDA receptor function. Because amygdala NMDA receptors play a significant role in many learned-fear behaviors, chronic ethanol-induced adaptations in these receptors may influence the expression of withdrawal anxiety.
Recent advances in understanding the neuroanatomy of anxiety behaviors have identified the amygdala as a central regulatory brain area. The lateral and basolateral subdivisions are the amygdala’s primary input areas where sensory information is extensively processed and forwarded to both the central amygdala and other ‘emotional’ forebrain regions. Because these subdivisions are functionally related and are closely juxtaposed anatomically, they will be referred as the lateral/basolateral amygdala (BLA) throughout this work. Inactivation or chemical lesions of the BLA disrupt learned fear measured in several different behavioral paradigms. For example, direct muscimol injections prior to training, but not after training, block the acquisition of learned fear (Wilensky et al., 1999). Lesions of the BLA also disrupt the ability of drug-associated cues to re-instate drug seeking behavior (Meil and See, 1997).

Analysis of neurotransmitter systems within the lateral/basolateral amygdala has shown that NMDA receptors play a unique role in fear-learning. NMDA receptor antagonists injected directly into the lateral/basolateral amygdala block the association of sensory cues with footshock in many fear-learning paradigms (Fanselow and Kim, 1994; Hatfield and Gallagher, 1995). Like these antagonists, ethanol can inhibit NMDA receptor function and can disrupt fear learning as well (Sonner et al., 1998), suggesting that the lateral/basolateral amygdala is an important site of ethanol action.

NMDA receptors are multimeric complexes containing distinct families of protein subunits. The NR1 subunit family is required for the formation of functional channels and is represented by a single gene theoretically encoding eight unique splice variants (Laurie and Seeburg, 1994). NR2 subunits produce channels similar to those found in native systems when co-expressed with NR1 (Williams et al., 1994) and are encoded by four separate genes (NR2A-D). Expression of the different NR2 subunits can confer unique pharmacologic and biophysical
properties to native receptors. Since the expression of NR2 subunits is both temporally and spatially regulated (Monyer et al., 1994), the characteristics of NMDA receptors vary both during development and from brain region-to-brain region. NR3 subunits share sequence similarity with other NMDA subunits and appear to function in a dominant negative manner when co-expressed in a receptor complex (Sucher et al., 1995). These subunits are highly expressed in embryonic CNS (Sucher et al., 1995) and appear to regulate the development of NMDA receptor-containing synapses (Das et al., 1998). NR3 subunit expression declines in adults to very low levels although substantial expression is still evident in the temporal lobe, including the amygdala (Ciabarra et al., 1995). Thus, a diversity of NMDA receptor subunits may help to determine the functional characteristics of these important channels.

Acute inhibition of NMDA receptor-gated currents by intoxicating concentrations of ethanol (Lovinger et al., 1989) has been demonstrated for numerous isolated neuronal preparations as well as for NMDA-mediated synaptic responses in several brain regions. Subunit composition appears to influence this interaction as well. For example, NR2A- or NR2B-containing receptors are relatively more sensitive to acute ethanol inhibition compared to those channels containing NR2C or NR2D when expressed in heterologous systems (Masood et al., 1994). Consistent with this, both ethanol inhibition and inhibition by the NR2B-selective antagonist decreases in cortical neurons as their time in culture increases (Lovinger, 1995). However, subunit-specific post-translational modifications and protein-protein interactions can also dramatically influence the acute ethanol sensitivity of NMDA receptors (Yaka et al., 2003). Thus, many factors may influence the interaction between ethanol and the NMDA receptor. This diversity is manifest frequently as variations in ethanol potency or efficacy across brain regions.
In addition to acute ethanol action, chronic ethanol exposure can also alter NMDA receptor function and subunit composition. Functional NMDA receptor binding is increased following chronic ethanol in several brain areas, including the hippocampus and cortex (Gulya et al., 1991). Chronic ethanol also enhances NMDA-mediated currents in medial septum neurons (Grover et al., 1998) although direct measures of chronic ethanol’s influence on NMDA channel function in native cells are not abundant in the literature. Due to the prominent role of NMDA receptors in amygdala-dependent behaviors, we have therefore focused chronic ethanol’s effects on native NMDA receptor pharmacology, function, and expression in rat lateral/basolateral amygdala. Portions of this work have appeared in abstract form.
Methods

Chronic ethanol administration.

All animal procedures were in accordance with the guidelines set forth by NIH animal care and use policy. Adult male Sprague-Dawley rats (Harlan, Inc.) were housed individually in an AAALAC-accredited facility and were kept on a 12-hr light/dark cycle. Animals were randomly placed into two treatment groups, those receiving a non-ethanol containing liquid diet (“Control” group) and those chronically exposed via an ethanol-containing liquid diet. These diets were commercially obtained (Bio-Serv Inc.; Frenchtown, N.J.) and are similar to that reported by Lieber & DeCarli (Lieber and DeCarli, 1989). ‘Chronic ethanol’ rats received the ethanol diet (4-6% v/v) for a total of 10-12 days. ‘Control’ rats were ‘yoked’ to the ethanol rats and received a volume of the control diet equivalent to the consumption by the ‘ethanol’ rats on the previous day. Both ‘control’ and ‘chronic ethanol’ rats gained a similar amount of weight during the liquid diet treatments (P>0.6, two-tail t-test): start weights were 132±10g for controls (n = 16) and 133±8g for chronic ethanol rats (n = 17); and, finishing weights were 195±10g and 203±11g for control and chronic ethanol rats, respectively. Water was given ad libitum and diet intake monitored daily. ‘Chronic ethanol’ rats consumed 11.2±0.4g/kg/day ethanol and were sacrificed while intoxicated. Where it was measured, blood ethanol concentrations at the time of sacrifice were 153±16 mg/dl (n=17).

Brain slice preparation and neuronal isolation.

To prepare brain slices, rats were anesthetized with isoflurane and decapitated. Tissue slices containing the BLA were prepared as previously reported (McCool et al., 2003). After preparation, slices were stored in oxygenated Ringer’s solution for up to 5 hours. Individual neurons were isolated from tissue by incubation of a single slice for 20 minutes at 37°C in
oxygenated Ringer’s solution containing 0.5-0.75 mg/ml Pronase protease (CalBiochem, San Diego, California, USA). Digested tissue was passed through a series of fire polished Pasteur pipettes; and, neurons were allowed to settle on tissue culture-treated plastic dishes or coverslips. 150 mg/dl ethanol was added to brain slices from chronic ethanol exposed rats during storage to prevent withdrawal in vitro. In a separate set of experiments, we determined that a ~2.5 hour in vitro ethanol exposure of ‘control’ brain slices did not affect maximal NMDA responses. This time was chosen since it represents the average time slices were stored during a typical experiment. Using 1 mM NMDA, current densities were 27±4 pA/pF for slices incubated without ethanol (n = 18) and 24±7 pA/pF for slices incubated with 150 mg/dl ethanol during storage (n = 6; P>0.1, t-test). Ethanol was absent from chronic ethanol cells during the enzymatic treatment, neuronal isolation, and recording periods.

Electrophysiology.

The whole cell patch clamp technique was performed on acutely dissociated neurons that were continuously perfused with a HEPES-buffered saline (HBS) solution consisting of: 150 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 2.5 mM CaCl₂, 1.0 mM MgCl₂, 10 mM D-glucose; pH 7.4 with NaOH, osmolality 320 mmol/kg adjusted with sucrose. Drugs were diluted from concentrated stocks into HBS lacking MgCl₂ and containing only 0.2 mM CaCl₂ along with 0.5-1 µM tetrodotoxin. This solution was applied within 100 µm of the cell using a linear array of fused silica tubes (150 mm I.D., Hewlett Packard) mounted on a manipulator. A Cs⁺-based internal solution (in mM: 120 CsCl, 10 HEPES, 11 EGTA, 1 CaCl₂, 4 Mg-ATP; pH 7.2 with CsOH, osmolality 305 mmol/kg adjusted with sucrose) was used in the patch electrode.

Recordings were done at room temperature using an Axopatch ID amplifier (Axon Inst.) in voltage-clamp mode as previously described (Hamill et al., 1981). Whole-cell capacitance
and series resistance were determined by fits of the capacitive transients during square-wave voltage steps using standard software procedures contained within pClamp 7.0 software (Axon Inst.) and monitored throughout the recordings. Series resistance and capacitance were compensated manually.

Current amplitudes were measured from the apparent peak of the response and are reported as values standardized to the whole cell capacitance (in pF) derived from fits to a square-wave depolarization (pClamp 7.0, Axon Inst.). Concentration-response relationships were derived using commercially available software (Prism, GraphPad Software) where data were fit to the standard logistic equation:

\[ Y = Y_{\text{MAX}} \left(1 + 10^{\left(-\text{LogEC}_{50} - X / \text{Hill-slope}\right)}\right) \]

Summarized data is reported as the mean ± SEM.

**Real-time RT-PCR**

Total RNA was isolated from the lateral/basolateral amygdala of individual control and chronic ethanol rats or from whole forebrain using affinity chromatography (RNeasy Mini Kit; Qiagen). Contaminating genomic DNA was removed by digestion with DNase I according to the manufacturer’s instructions. RNA concentrations were determined using fluorescent detection (RiboGreen; Molecular Probes). The reverse transcription (RT) reaction was performed on 4-8ng/µl total RNA using random hexanucleotides as previously described (McCool and Farroni, 2001). Real-time PCR was performed on cDNA products using the TaqMan detection method (reviewed in (Giulietti et al., 2001)) and a Prism 7000 Sequence Detection System (Applied Biosystems). Taqman ‘Universal PCR Mix’ (Applied Biosystems) containing Taq DNA polymerase, dNTPs (+dUTP), and buffers was used according to manufacturers directions. Pilot experiments demonstrated that 0.9µM primers and 0.25µM probe produced the largest change in fluorescence during the
course of a PCR reaction for all gene products examined. Primer and probe combinations were designed using PrimerExpress software (version 3.0, Applied Biosystems) and rat sequences available on public databases (GenBank). For the rat NR1 subunit, regions around the various splice sites were excluded from consideration. Similarly, cDNA sequences for the NR2 or NR3 family were compared and regions greater than 85-90% similar were not included for primer/probe design. Probes were labeled with 5’FAM and 3’TAMRA. Primers and probes for each NMDA subunit mRNA and for the ubiquitous gene glyceraldehyde phosphate dehydrogenase (GAPDH) are shown in Table 1.

The PCR reaction consisted of initial incubations at 50°C for 2min followed by 95°C for 10min; steps were 40 cycles of 95°C for 15 sec to melt DNA duplexes followed by 60°C 1min for annealing and product formation. Fluorescence was measured at the end of the annealing/extension step. Background fluorescence, defined as the fluorescence during early portions of the PCR reaction before any substantial accumulation of PCR product, was subtracted from each reaction. Ct values were defined as an arbitrarily ‘cut-off’ change in fluorescence above background, typically 0.1 to 0.3 log-units depending upon the absolute level of fluorescent intensity, during the log-linear phase of the PCR reaction.

The ‘relative standard curve’ method (Johnson et al., 2000) was used to compare expression levels of mRNAs between the control and chronic ethanol samples. For this, serial dilutions of cDNAs prepared from total forebrain RNA were subjected to real-time PCR with gene-specific primer/probe combinations to establish a standard curve. Real-time PCR reactions on cDNAs prepared from control or chronic ethanol total RNA samples were performed at the same time; and, Ct values of these samples were related to
'ng Forebrain RNA’ equivalents using the linear relationship between the log(ng Forebrain RNA) in a particular standard curve reaction and its corresponding C\textsubscript{T} value (see Fig. 5). Each gene’s ‘ng Forebrain RNA’ equivalent in a given sample was normalized to the relative expression level GAPDH in that same sample.

**Single-Cell RT-PCR**

Single-cell RT-PCR on dissociated basolateral amygdala neurons was performed as described previously (McCool and Farroni, 2001). For some experiments, individual neurons were harvested by simple aspiration into a borosilicate glass pipette containing ~5µl of ribonuclease-free water and ribonuclease inhibitor (0.1U/µl). In other experiments, neurons were harvested following electrophysiology; in these cases, ribonuclease inhibitor (0.1U/µl) was added to the recording internal solution described above. There were no apparent differences in the NR2 expression profile between neurons harvested in water versus those harvested after electrophysiology.

For the polymerase chain reaction, ‘hot-start’ was used to initiate the reaction by addition of Taq polymerase following a 2 minute heat denaturation. Samples were subjected to 40 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, Massachusetts, U.S.A.). Amplicons were analyzed by agarose gel electrophoresis using ethidium bromide fluorescence. Oligonucleotide primers were derived from unique sequences to specifically amplify individual NR2 subunits (Porter et al., 1998), the 67kD form of Glutamic acid decarboxylase (GAD67) (Ceranik et al., 1997), or glyceraldehyde phosphate dehydrogenase (GAPDH) (Salazar et al., 1999). All oligonucleotides were obtained from commercial sources (Sigma Genosys, The Woodlands, Texas, U.S.A.).
To statistically examine the largely qualitative scRT-PCR data, a binomial nomenclature (‘0’ = no expression, ‘1’ = expression detected) was used to describe results for each gene in any given cell. Arrays were generated for the expression of a given mRNA derived from each treatment and were compared (control versus chronic ethanol) using resampling methods contained within the “Resampling Stats Add-in” for Excel (Resampling Stats Inc., Arlington VA, USA). Briefly, if treatment had an effect on the expression of a given gene, the difference between the fraction of cells expressing the gene (number of ‘1’s divided by total number of neurons tested) in each treatment group would be relatively large; and, the magnitude of this difference would occur only rarely in artificially generated arrays where the total number of ‘1’s and ‘0’s as the original data set were randomly assigned between the treatment groups. The distribution of the differences between treatments for these random arrays was used to discern the ‘probability’ of obtaining the difference derived from the real data. In our case, we used the original data to generate 10,000 random arrays. A difference value in the real data that was represented by equal or larger magnitudes in less than 500 out of 10000 simulated arrays would be assumed to predict a P<0.05 (see http://www.resample.com/content/software/excel/userguide/index.shtml for methodological details).
Results

*Chronic Ethanol Enhances NMDA Efficacy but Not Potency*

To assess NMDA receptor function in control (n = 7 individuals) and CE rats (n = 7 individuals), we established NMDA concentration-response relationships in isolated lateral/basolateral amygdala neurons (Figure 1A). We normalized currents (in pA) to apparent cell capacitance (in pF) to reduce cell-to-cell variability. Neither whole-cell capacitance (18±1pF versus 19±1pF, P>0.5 with two-tail t-test) nor access resistance (24±1MΩ versus 24±2MΩ, P>0.9 with two-tail t-test) was significantly different between control neurons (n=39) and chronic ethanol (CE) neurons (n=43), respectively. In the presence of 3µM glycine, current densities from 30µM to 1mM NMDA were significantly larger in neurons isolated from CE rats when compared with controls (Fig. 1B, P<0.05 at each concentration, two-tailed t-test). For example, at the maximal concentration tested (1mM), chronic ethanol NMDA currents were 56±8 pA/pF (n = 21 neurons) while control currents were 35±6 pA/pF (n = 16 neurons). A similar trend was noted for comparisons between current amplitudes without normalization, with current amplitudes with 0.3mM from control neurons (532±94pA) being significantly smaller (P<0.05) than amplitudes from chronic ethanol neurons (868±125pA). In one experiment, neurons from a chow-fed rat exhibited a 1mM NMDA/3µM glycine current density (33±9 pA/pF, n = 3) that was almost identical to control rats, suggesting that the differences between control and CE current density was due to the ethanol exposure in the latter group and that the liquid diet had little impact on ‘basal’ NMDA receptor function. Despite the elevated NMDA receptor function in CE neurons, the apparent affinity and Hill slope defined by the concentration-response relationship were not significantly different between treatment groups (Fig. 1B). In cells where complete concentration-response relationships were defined, the log
[EC$_{50}$] values were -4.1±0.1 for control neurons (n = 14, equivalent to 75µM) and -4.1±0.1 (n = 18, 68µM) for CE neurons (P>>0.05, t-test with Welch’s correction). For these same neurons, the Hill slopes, 1.3±0.1 for controls and 1.5±0.1 for chronic ethanol cells, were also not significantly different.

More detailed examination of the current densities for 300µM NMDA from individual neurons in each treatment group (Fig. 1C) revealed that chronic ethanol may have enhanced the average current density by increasing the number of neurons with densities >50pA/pF. Although there were neurons with comparable densities in the control group (n =3 out of 21 or 14%), there were substantially more of these cells (n=9 out of 19 or 47%) in the chronic ethanol group. Thus, while the range of current densities was similar between treatment groups (5-97pA/pF for control neurons versus 12-126pA/pF for chronic ethanol neurons), the median current density for chronic ethanol neurons (50pA/pF) was over twice that for control neurons (23pA/pF).

**Chronic Ethanol Does Not Alter the Acute Ethanol Sensitivity of NMDA Receptors**

As in many other preparations, NMDA-gated currents in acutely isolated lateral/basolateral amygdala neurons were inhibited by acute application of ethanol (Fig. 2A). However, chronic ethanol ingestion did not appear to alter this acute sensitivity. Using 30mM ethanol, NMDA currents were inhibited by 18±7% in control neurons (n=4, Fig. 2B) and by 15±2% (n=9) in chronic ethanol neurons (P>>0.1, t-test). Likewise, 100mM ethanol inhibited NMDA currents by 51±7% (n=10) and 42±5% (n=11) in control and chronic ethanol neurons, respectively. Although there was an apparent trend for reduced inhibition at this higher ethanol concentration, the differences between treatment groups were not significantly different. The distribution of the individual percent inhibition values at 100mM ethanol was also similar.
between groups (20-81%, median=47% for control neurons versus 21-71%, median=41% for chronic ethanol neurons).

**Chronic Ethanol Increases Inhibition by Ifenprodil**

The non-competitive antagonist ifenprodil (10µM) substantially inhibited NMDA-gated currents elicited by 100µM NMDA and 3µM glycine in a majority of neurons tested. 10µM ifenprodil represents a concentration that maintains some selectivity for the inhibition of NR2B-containing NMDA receptors relative to receptors composed of other subunits (Williams, 1993). In many neurons, ifenprodil inhibition was slow to reach its maximal extent during application of an NMDA/ifenprodil admixture, as has been described for the action of this antagonist (Williams, 1993). There were also a large number of neurons where ifenprodil inhibition did not have this slow-onset but was none-the-less substantial (see Figure 3 & 7 for some examples). To insure we examined ifenprodil inhibition at its fullest extent, we compared the percent inhibition at the apparent steady-state current level (“I$_{SS}$”), approximately 4 seconds after the peak of the current response (see Fig. 3). Ifenprodil inhibited steady-state current by 29±4% in control neurons (n=16) and by 46±5% (n=18) in chronic ethanol neurons (P<0.05, t-test). In addition of steady-state current amplitudes, peak-to-steady-state current ratios are often used to describe ifenprodil inhibition and can provide a better appreciation for its slow-onset. In control neurons, I$_{Peak}$/I$_{SS}$ ratios were 1.5±0.1 in the presence of 10µM ifenprodil and significantly increased to 2.0±0.4 in CE neurons (P<<0.01, two-tailed t-test). Both results strongly suggest that chronic ethanol ingestion increased the contribution to functional receptors by the NR2B subunit.

**Influence of Extracellular Calcium on Current Kinetics: Interactions with Chronic Ethanol**

Because chronic ethanol appeared to influence the NR2 subunit composition of NMDA receptors, we next examined the impact of chronic ethanol exposure on the effects of
extracellular calcium since NR2 subunits can also influence calcium-dependent current inactivation (Krupp et al., 1996). With low extracellular calcium (0.2mM), currents evoked using an EC$_{50}$ concentration of NMDA plus 3µM glycine did not appreciably inactivate during the agonist exposure (see Figs. 1-3). However, increasing the extracellular Ca$^{2+}$ (Ca$_{o}^{2+}$) concentration from 0.2mM to 2mM substantially increased the apparent rate of current inactivation during the agonist exposure (Fig. 4A). Since high Ca$_{o}^{2+}$ attenuated the ‘steady state’ portion of the current (I$_{SS}$) much more than the peak (I$_{Peak}$) current amplitude, we specifically examined the effects of 2mM Ca$_{o}^{2+}$ on I$_{SS}$. The inhibition of I$_{SS}$ in control neurons (58±5%, n = 6, Fig. 4B) was significantly larger than in chronic ethanol neurons (35±2%, n = 12, P<0.01 in two-tail t-test). Current inactivation described by the I$_{Peak}$/I$_{SS}$ ratio during exposure to 2mM Ca$_{o}^{2+}$ was also increased in control neurons relative to CE neurons, as demonstrated by the average current traces from control and chronic ethanol neurons in Figure 4C. The I$_{Peak}$/I$_{SS}$ ratio in presence of 2mM Ca$_{o}^{2+}$ was 2.15±0.18 in control neurons (n = 6, Fig. 4D) while the ratio in CE neurons was 1.71±0.09 (n = 12, P<0.05, two-way t-test). In contrast, the ratios in 0.2mM Ca$_{o}^{2+}$ for control neurons (1.39±0.09) was not different from chronic ethanol neurons (1.35±0.05), indicating the differences between the I$_{Peak}$/I$_{SS}$ ratios of control and chronic ethanol neurons in 2mM Ca$_{o}^{2+}$ was likely specific to permeation of calcium through the NMDA channels.

**Chronic Ethanol Alters the Expression of Some NMDA Receptor Subunit mRNAs**

Since our functional data indicated that chronic ethanol could influence NMDA receptor levels and alter NR2 subunit contributions, we examined regulation of mRNA expression levels as a potential molecular mechanism. Given that the lateral/basolateral amygdala is a relatively small brain region, comparisons between control and chronic ethanol animals were achieved
using real-time RT-PCR with ‘TaqMan’ detection (Giulietti et al., 2001). Total forebrain RNA was used with the ‘relative standard curve’ method (Johnson et al., 2000) to quantify gene expression within individual samples. An example of the real-time method is shown in Figure 5A. Importantly, the relationship between the log[ng total Forebrain RNA] put into the PCR reaction and the relative expression level, represented by the ‘Cycle Threshold’ (or $C_T$, see Methods), of each gene product was linear over a wide range concentrations (Fig. 5B). This insured that $C_T$ could be directly related to the amount of the mRNA in the original sample.

Using this real-time RT-PCR method, we compared relative expression levels of each NMDA receptor subunit mRNA in control and chronic ethanol rats. Expression levels of NMDA subunits were normalized to levels of the ubiquitous gene product for glyceraldehyde phosphate dehydrogenase (GAPDH) in each sample. Importantly, GAPDH mRNA expression was not significantly different between control and chronic ethanol samples (Figure 6A).

Although all NMDA subunit mRNAs were detected in the BLA, neither NR2 nor NR3 subunit mRNA levels were significantly different between the treatment groups (see Fig. 6C and Table 2). In contrast, NR1 subunit mRNA levels were significantly increased by chronic ethanol exposure (Fig. 6B; $P<0.05$, t-test). Relative expression levels of NR1 mRNA were $22.0\pm1.4$ for control samples and $28.0\pm1.5$ in BLA exposed to chronic ethanol, a ~25% increase. The relative expression level of NR1 as well as the remaining subunits was not significantly correlated with absolute amount of ethanol consumed by an individual rat (not shown).

Chronic Ethanol Does Not Alter the NR2 Expression Profile of Individual Amygdala Neurons

Since the expression of NR2C and NR2D was detected in total lateral/basolateral amygdala RNA and was not anticipated, we subsequently examined NR2 expression in single
isolated neurons to identify the cellular source for these various mRNAs. As shown in Fig. 7A&B, many neurons expressed multiple NR2 subunit mRNAs, with NR2A and NR2B mRNAs being the predominant species detected with this method (Fig. 7A&B). Although we attempted to examine isolated neurons with morphological characteristics consistent with glutamatergic projection neurons (McDonald, 1982), we confirmed the cell phenotype in these molecular studies by using GAD65 mRNA expression as a marker for GABAergic interneurons. In 12 control neurons, 10 cells did not appear to express GAD (Fig. 7C). Three out of 10 of these GAD− control neurons (30%) expressed NR2A subunit mRNA alone. 5 out of the remaining 7 neurons (50%) expressed both NR2A and NR2B. Interestingly, one control neuron expressed NR2A, NR2B, and NR2D (10%); while the final neuron (10%) expressed all known NR2 subunits. Chronic ethanol exposure did not appear to qualitatively alter this pattern of expression. Fourteen out of 17 ‘chronic EtOH’ neurons yielded signal for NR2 expression. 12 of these neurons appeared to be GAD+. 5 of the 12 NR2+/GAD− chronic ethanol neurons (42%) expressed only NR2A (Fig. 6D); while 1 out of 12 (8%) expressed only NR2B. Of the remaining six neurons, 5 ‘chronic EtOH’ neurons (42%) expressed both NR2A and NR2B; while a single NR2A/B+ neuron (8%) also expressed NR2D. To provide some quantitative comparisons of these largely qualitative data, we used re-sampling techniques (see Methods) but did not detect any significant statistical differences between the NR2 expression profiles of the control and chronic ethanol groups when the NR2 mRNAs were considered individually.

Our morphological selection process limited the number of GAD+ neurons examined, preventing us from making any specific measures of chronic ethanol on NR2 expression in this cell population. However, our preliminary results indicate that the NR2 expression profile of GAD+ neurons may be distinct from that found in the GAD− population. Although two out of 6
GAD$^+$ neurons from all experimental groups expressed only NR2A and NR2B, the expression profile in the remaining four GAD$^+$ neurons was heterogeneous and consisted of NR2A+D, NR2A+B+C, NR2A+C+D, and NR2A+B+D. Thus the GAD$^+$ population of neurons is the most significant source for NR2C and 2D expression in the lateral/basolateral amygdala.
Discussion

One of the major findings of our study was that chronic ethanol exposure resulted in a pronounced functional increase in NMDA receptors expressed by lateral/basolateral amygdala neurons. Since NMDA receptors in this brain region are important both for fear responses to unconditioned stimuli (Adamec et al., 1999) and for conditioned ‘fear-learning’ (Miserendino et al., 1990), chronic ethanol-induced increases in NMDA receptor function may help ‘identify’ the withdrawal state as an aversive event and ultimately help accentuate drug-seeking behaviors. It will be particularly interesting to correlate the time course of withdrawal anxiety and the subsequent development of drug-seeking with the functional properties of lateral/basolateral amygdala NMDA receptors.

A second important finding of our studies was that chronic ethanol exposure altered the pharmacological properties of amygdala NMDA receptors. However, acute ethanol sensitivity was not changed, suggesting little or no tolerance to ethanol by amygdala NMDA receptors in this system. The impact of chronic ethanol exposure on acute ethanol sensitivity on NMDA receptors has varied in the literature. For example, the acute ethanol sensitivity of NMDA-induced neurotoxicity appears diminished following chronic ethanol exposure of cultured cerebellar granule cells (Cebere et al., 1999), while chronic ethanol did not attenuate the acute ethanol inhibition of NMDA receptor-dependent intracellular calcium increases in these same neurons (Iorio et al., 1992). Similarly, acute ethanol inhibition in acutely dissociated medial septum/diagonal band neurons is attenuated following chronic ethanol (Grover et al., 1998). However, acute ethanol inhibition of amygdala NMDA currents (this study) and NMDA currents in hippocampal slices or cultured neurons (White et al., 1990) were not altered by chronic ethanol exposure. While different end-point measures might explain the disparate findings in
cultured cerebellar neurons, the divergent findings in the latter electrophysiology studies may indicate brain region-specific tolerance of NMDA receptor acute ethanol inhibition following chronic ethanol exposure. Furthermore, it is unclear how absolute amounts of ethanol exposure or the total length of exposure might have influenced these studies.

In contrast to acute ethanol, chronic ethanol ingestion did appear to influence the functional contribution by specific NMDA receptor subunits. The inhibition of amygdala NMDA currents by the non-competitive, NR2B-specific antagonist ifenprodil was enhanced in neurons from ethanol-exposed animals. This increase in NR2B function is also reflected by the apparent increase in $I_{\text{Peak}}/I_{\text{SS}}$ ratio in the presence of ifenprodil. Although we used an ifenprodil concentration that has maximal effects on NR2B (Williams, 1993), we can’t exclude the possibility that combinations of NR2B with other NR2 subunits might reduce the apparent efficacy of ifenprodil and mask chronic ethanol-induced increases in these subunits (see (Blevins et al., 1997)). Our approach of combining NR2 expression profiling using single-cell RTPCR with electrophysiology did in fact demonstrate the presence of multiple NR2 subunit mRNAs in many neurons. Regardless, these ifenprodil findings are supported by the alterations in calcium-dependent inactivation following chronic ethanol exposure. Alterations in the peak-to-steady-state ratio may further suggest that calcium-dependent inactivation may be decreased following chronic ethanol exposure. The influence of chronic ethanol on absolute current amplitude in different extracellular calcium environments could also be interpreted as a relative increase in $\text{Ca}^{2+}$-permeability (see (Plant et al., 1997)). Regardless of the interpretation, NR2 subunits can dramatically influence $\text{Ca}^{2+}$ entry through NMDA receptors (Blevins et al., 1997), with the rank order of permeability for NMDA-gated channels containing different NR2 subunits being $\text{NR2B}>\text{NR2A+NR2B}>\text{NR2A}$. These results together suggest that the larger current amplitudes
in the presence of ‘high’ extracellular Ca\(^{2+}\) for chronic ethanol neurons are consistent with an increased functional contribution by ifenprodil-sensitive NR2B-containing channels. Although the ultimate consequences of enhanced Ca\(^{2+}\) entry or decreased calcium-dependent inactivation following chronic ethanol ingestion are yet to be directly addressed, NR2B-containing amygdala NMDA receptors participate in fear-learning behaviors (Rodrigues et al., 2001). Furthermore, calcium entry through NMDA receptors is essential for LTP-like increases in synaptic efficacy (Regehr and Tank, 1990) that can ultimately result in increased ‘fear-learning’ during or following chronic ethanol ingestion. Our results therefore suggest important consequences are likely to be related to functional adaptations of lateral/basolateral amygdala NR2B subunit.

Alterations in functional contributions by different NR2 subunits following chronic ethanol exposure led us to examine the influence of gene expression as a potential contributor to receptor adaptation. However, real-time RT-PCR measures of NR2 subunit mRNA expression failed to detect any significant alterations in these subunits during chronic ethanol exposure. This suggests that the altered functional contributions by the NR2B subunit during chronic ethanol are not mediated by changes in NR2B mRNA transcription or stability. Recently, acute ethanol has been shown to influence the phosphorylation status of the NR2B subunit (Yaka et al., 2003). Since phosphorylation of this subunit can also have a profound impact on receptor function (Yaka et al., 2002), it is entirely possible that NR2B adaptation in lateral/basolateral amygdala is governed by non-transcriptional mechanisms. Regardless, the up-regulation of NR1 subunit mRNA levels during chronic ethanol may help accentuate chronic ethanol-dependent up-regulation of receptor function. Our molecular findings further suggest that the molecular mechanisms related to NMDA receptor adaptation in lateral/basolateral amygdala may be distinct from those in the cerebral cortex where chronic ethanol up-regulates functional receptors.
(Gulya et al., 1991) without altering NR1 subunit mRNA expression (Morrow et al., 1994). This contrast also highlights the diversity of region-specific transcriptional and post-transcriptional mechanisms responsible for chronic ethanol-induced alterations in NMDA receptor expression and function.

To our knowledge, this is the first report of NR2C, NR2D, NR3A, and NR3B mRNA expression in lateral/basolateral amygdala. Although the absolute levels of these subunits cannot be defined using the methods used here, we can consider their expression level relative to forebrain without normalizing to GAPDH. Combining data from control and chronic ethanol samples (n=17), NR2C expression per ng total lateral/basolateral RNA was equivalent to 5.6±1.2ng forebrain RNA; while NR2D expression was equivalent to 0.7±0.1ng forebrain RNA per ng lateral/basolateral RNA. Similarly, NR3A expression per ng total lateral/basolateral RNA was similar to that found in 2.5±0.4ng forebrain RNA; while, NR3B expression in one ng total lateral/basolateral amygdala RNA is equivalent to 1.4±0.3ng forebrain RNA. These results indicate that NR2C and NR3A transcripts are relatively more abundant in the lateral/basolateral amygdala compared to the forebrain, while NR2D and NR3B expression is approximately equivalent.

Like the real-time RT-PCR experiments, our single-cell RT-PCR data was unable to identify any significant shift in NR2 expression profile following chronic ethanol. These results support our more quantitative findings with real-time measures. In addition, several general findings are of some interest. First, we found that the majority of neurons examined by scRT-PCR were GAD-negative, suggesting that our physiological examinations were performed primarily upon GAD⁺, presumably projection neurons. Second, although our neurons were taken from adult rats (ca. 200g), most GAD⁺ neurons expressed both NR2A and NR2B mRNAs,
indicating the NR2B-to-NR2A transition evident in other brain regions during the juvenile to adult phase of development (Monyer et al., 1994) may not take place in the BLA or be incomplete in the animals examined here. We were also surprised to find that a small population of GAD-negative neurons (approximately 20% across all treatment groups) appeared to express NR2D subunit mRNA. While previous work in other laboratories (Monyer et al., 1994) did not indicate any significant expression of this subunit in this brain region, it is possible that the modest-to-low levels of expression noted in our real-time experiments and the relatively small number of neurons identified by scRT-PCR might have prevented detection by less sensitive methods. Finally, despite our attempts to select neurons that morphologically resemble projection neurons, GAD expression was none-the-less detected in 8 out of 42 cells (19%) examine be single-cell RT-PCR. Importantly, a subset of the GAD+ presumed interneurons appeared to be the primary source of NR2C mRNA expression in the lateral/basolateral amygdala. Regardless, the GAD+ cells sampled by our molecular studies may represent the distinct class of relatively ‘large, pyramidal’ interneurons identified in previous anatomical studies (McDonald, 1982). However, the number of GAD+ interneurons with this phenotype should be quite small relative to the large number of GAD-negative projection neurons. This in turn suggests that the procedure for acutely separating individual neurons from tissue may somehow bias for the isolation of GAD+ cells.

In conclusion, chronic ethanol ingestion enhances lateral/basolateral amygdala NMDA receptor function. While this enhancement appears to involve alterations in NR2 subunit content of the functional receptor complex, we find no indication that pronounced alterations in the NR2 subunit mRNA expression plays any role in these adaptations. Since lateral/basolateral amygdala NMDA receptors can regulate a number of important behaviors, from cue-specific fear
learning to drug-seeking, it is likely that functional adaptations of these receptors may play a significant role for many of the behaviors manifest during and/or following chronic ethanol exposure.
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Footnotes:

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Legends for Figures

Figure 1. Chronic ethanol ingestion enhances NMDA receptor current density in acutely isolated lateral/basolateral amygdala neurons. A. Examples of current traces from control (A1) and chronic ethanol (A2) neurons. Currents were elicited by application of different NMDA concentrations (10µM to 1mM) along with 3µM glycine. Densities in these particular cells were similar to the mean density from each treatment group. Calibration bars: y = 200pA, x = 1 sec. B. Ensemble concentration-response data indicates that enhanced NMDA efficacy following chronic ethanol (○, 56.3±8.0pA/pF versus 34.7±6.4pA/pF in control ■ at 1mM NMDA) was not accompanied by a substantial alteration in apparent EC₅₀ (68µM for chronic ethanol neurons and 75µM for control neurons). NMDA current density from chow-fed animals (□) was nearly identical to that found in control liquid diet (33±9pA/pF, n=3). * -- indicates significant difference (P<0.05, two-tailed t-test) between control and chronic ethanol current densities. C. A scatter plot of current densities from individual neurons indicates that there may be an ethanol-sensitive population of neurons from the lateral/basolateral amygdala. Lines through the groups of points indicate the population mean. The dashed box represents the middle 50% of the population from control neurons (■). Note the apparent increase in the number of neurons with densities >50pA/pF following chronic ethanol ingestion (○).

Figure 2. Chronic ethanol ingestion does not alter the apparent sensitivity of NMDA responses to acute ethanol. A. Examples of NMDA/glycine responses with and without 100mM ethanol from a control rat. Drug applications were manually controlled and of different durations for this set of traces. Calibration bars: y = 200pA, x = 1 sec. B. Summary of percent inhibition measures for 100mM and 30mM ethanol indicate that chronic ethanol does not alter acute
ethanol sensitivity at either ethanol concentration. Note that there was a trend for 100mM ethanol inhibition to be reduced following chronic treatment but that the effect was not statistically significant.

Figure 3. Chronic ethanol exposure enhances inhibition of NMDA current responses by the NR2B-selective antagonist ifenprodil. A&B. Examples of ifenprodil inhibition (10µM) of NMDA/glycine currents in a control neuron (A) and a chronic ethanol neuron (B) indicate an increase in ifenprodil inhibition following chronic ethanol ingestion. The steady state (I_{SS}) of the current trace in A is indicated. Calibration bars: y = 100pA (A) or 200pA (B), x = 1 sec. C. Summary of the ifenprodil inhibition at I_{SS} reveal a significant difference (★, P<0.05) between control (29.4±3.9%) and chronic ethanol neurons (42.2±4.4%).

Figure 4. Chronic ethanol ingestion decreases calcium-dependent inactivation of NMDA-mediated currents. A. Example of NMDA/glycine currents in the presence of either 0.2mM or 2mM extracellular Ca^{2+}. Note the distinct inactivation kinetics in each trace, represented by a more pronounced decrease in current amplitude at I_{SS} compared to I_{Peak}. Calibration bars: y = 200pA, x = 1sec. B. Percent inhibition of I_{SS} when switching from 0.2mM Ca_{o}^{2+} to 2mM Ca_{o}^{2+} is significantly larger (★ = P<0.05, t-test) in control neurons (58.0±5.2%) compared to chronic ethanol neurons (35.2±3.0%). C. Maximal NMDA/glycine current amplitudes with 2mM Ca_{o}^{2+} from control (8 traces) and chronic ethanol neurons (12 traces) were averaged. To illustrate the decrease in current inactivation following chronic ethanol, individual traces within each treatment group were subsequently scaled to this overall average. Note that the noise in the average control trace is larger than the average chronic ethanol trace because there were fewer
traces and these traces were generally smaller (-313±58pA versus -596±107pA). Calibration bar: x = 1sec. D. Summary of $I_{\text{Peak}}/I_{\text{SS}}$ ratio for both 0.2mM and 2.0mM extracellular $\text{Ca}^{2+}$ indicates that chronic ethanol ingestion significantly ($\star$ -- $P<0.05$, t-test) decreases the $I_{\text{Peak}}/I_{\text{SS}}$ ratio. Ratios were 2.1±0.2 for control neurons and 1.7±0.1 in chronic ethanol neurons.

Figure 5. Real-time RT-PCR can measure NR subunit mRNAs in lateral/basolateral amygdala. A. Example of real-time PCR reactions using primer/probe combinations for the NR2B subunit (□) and GAPDH (●) in total lateral/basolateral amygdala RNA from an individual rat. Average fluorescent signals from three independent reactions are shown; standard error bars are present but are hidden beneath symbols. $C_T$ values for each reaction were derived at 0.2 fluorescent units (dashed line). Average $C_T\pm$SEM is indicated for each gene product. B. $C_T$ values versus log(ng forebrain RNA) from different NR subunit and GADPH primer/probe combinations. The data demonstrate that the relationships between starting quantity of RNA and $C_T$ value are linear over a large range of RNA concentrations, including those used to measure relative expression in control and chronic ethanol samples (see Figure 6). The correlation coefficient for each combination is $\geq0.99$. These linear relationships were used to express the $C_T$ value of each NMDA subunit or GAPDH in control and chronic ethanol samples as an ‘ng Forebrain RNA’ equivalent.

Figure 6. Chronic ethanol ingestion enhances the expression of NR1 subunit in total RNA without affecting NR2 subunit levels. A. Chronic ethanol ingestion did not alter the expression of GAPDH mRNA levels in lateral/basolateral amygdala. GAPDH ‘ng Forebrain RNA’ equivalents were 1.9±0.2 for control animals ($n = 8$) and 1.9±0.3 for chronic ethanol animals ($n =$
9). The GAPDH equivalents calculated for each sample in each set of reactions were used to normalize NR subunit expression and reduce the impact of systematic errors in RNA quantification, variability in RNA integrity between samples, and experimenter error. **B.** NR1 subunit expression was significantly increased (*, p<0.05, two-tail t-test) in chronic ethanol samples (n = 8) compared to control (n = 6). Normalized expression levels were 22.0±1.4 for controls and 28.0±1.5 for chronic ethanol samples. This represents a ~25% increase in NR1 expression during chronic ethanol. **C.** Expression levels of NR2B were not affected by chronic ethanol ingestion. Normalized expression was 20.2±1.8 in control samples (n=8) and 19.4±2.0 in chronic ethanol samples (n=9). Chronic ethanol did not influence expression of any other NR2 subunit or of the NR3 subunits (see Table 2).

Figure 7. Chronic ethanol ingestion does not alter the NR2 expression profile of acutely isolated lateral/basolateral amygdala neurons. **A.&B.** Isolated neurons from control and chronic ethanol animals were ifenprodil sensitive and contained both NR2A and NR2B mRNAs. The profile of ‘cell A’ also indicated faint NR2D expression. NR2B was the only subunit detected in ‘Cell C’; note that slow-onset ifenprodil inhibition was also marked in this cell. Calibration bars: y = 100pA (cells A, B, D) or 200pA (cell C), x = 1 sec. **C.&D.** Summary of single-cell RT-PCR NR2 expression profiles for neurons isolated from control (C) and chronic ethanol (D) rats. While there was a decline for some subunit mRNAs during ethanol exposure, this treatment did not have any significant effects on NR2 expression profile (see text for details).
Table 1. Primers and Probes Used for Real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CGGATTTGGCCGTATTGG</td>
<td>CAATGTCCACTTTGTCAACAAGAGAA</td>
<td>CGCCTGGGTACCAGGGGCTGC</td>
</tr>
<tr>
<td>NR1-1b</td>
<td>GAATGATGGGGCGAGCTACTCA</td>
<td>ACGCTCATTTGTGATGGTCAGT</td>
<td>CCACAATGTGTCCGCTTGCCA</td>
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<tr>
<td>NR2A</td>
<td>GCTACACACCCTGCACCAATT</td>
<td>CACCTGGTAACCTCCTCAGTGA</td>
<td>TGCTCAATGGGAATGGGATGCA</td>
</tr>
<tr>
<td>NR2B</td>
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<td>CAGCTAGTCGGCTCTTTGGTT</td>
<td>AGACGCAAACCTCTAGGCGACAG</td>
</tr>
<tr>
<td>NR2C</td>
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<td>GTAAGCTGTCTTTATCGCCTTT</td>
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<tr>
<td>NR2D</td>
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<tr>
<td>NR3A</td>
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<td>NR3B</td>
<td>TCAGTAGACTGGCCCTTGATA</td>
<td>CCTCAGATCGCCTGGTTTTT</td>
<td>CTTCAGCCTGCTGTAACCTGACCC</td>
</tr>
</tbody>
</table>

a – Sequences for these cDNAs were obtained from the following GenBank entries: GAPDH, #M17701; NR1-1b, #U08263; NR2A, #NM_012573; NR2B, #U11419; NR2C, #NM_012575; NR2D, #NM_022797; NR3A, #U29873; and NR3B, #AF440691.
Table 2. Effect of Chronic Ethanol on Lateral/Basolateral Amygdala NMDA Receptor Subunit Expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Chronic EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1</td>
<td>22.0±1.4</td>
<td>28.0±1.5*</td>
</tr>
<tr>
<td>NR2A</td>
<td>19.1±1.9</td>
<td>16.4±1.5</td>
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<tr>
<td>NR2B</td>
<td>20.9±2.5</td>
<td>19.6±2.4</td>
</tr>
<tr>
<td>NR2C</td>
<td>48.5±3.0</td>
<td>48.6±2.9</td>
</tr>
<tr>
<td>NR2D</td>
<td>8.1±0.6</td>
<td>8.8±1.0</td>
</tr>
<tr>
<td>NR3A</td>
<td>44.8±3.1</td>
<td>46.5±2.1</td>
</tr>
<tr>
<td>NR3B</td>
<td>10.0±0.5</td>
<td>10.9±0.7</td>
</tr>
</tbody>
</table>

a – Expression levels are relative to total forebrain RNA and are normalized GAPDH expression in the same samples.

b – For NR1, NR3A, and NR3B, n = 6 control and 8 chronic ethanol animals. For the other subunits, n = 8 control and 9 chronic ethanol animals.

* – P<0.05, two-tailed t-test
Figure 1.
Floyd et al.
Figure 2
Floyd et al.

A

NMDA/Gly+ 100mM EtOH

100μM NMDA/3μM Gly

B

Acute Ethanol

Percent Inhibition

Chronic EtOH
Control

100mM 30mM

0 10 20 30 40 50 60

Control

100mM 30mM
Figure 3
Floyd et al.

A

Control Neuron

NMDA+
10μM Ifen

NMDA

B

Chronic EtOH Neuron

C

10μM Ifenprodil

Percent Inhibition (Iss)

Control

Chronic EtOH

*
Figure 4
Floyd et al.

A) 100μM NMDA/3μM Gly

+2.0mM Ca^{2+}

+0.2mM Ca^{2+}

B) 2mM Ca_{o}^{2+}

Percent Inhibition (I_{s})

Control Diet  Chronic EtOH

C) +2.0mM Ca^{2+}

Control

Chronic EtOH

I_{peak}

I_{ss}

D) 2mM Ca_{o}^{2+}

I_{peak}/I_{ss} Ratio

Control Diet  Chronic EtOH
Figure 5
Floyd et al.

A

Fluorescent Units

Cycle #

GAPDH (y=27.3-3.1x; R =1.00)

NR2C (y=30.9-3.3x; R =0.99)

NR2B (y=27.4-3.7x; R =1.00)

NR3B (y=33.7-3.6x; R =1.00)

B

Threshold Cycle (C_t)

NR1 (y=27.2-3.6x; R^2=0.99)

NR3A (y=33.1-3.8x; R =1.00)

NR2A (y=29.4-3.6x; R =0.99)

NR2D (y=35.0-3.8x; R^2=0.99)

NR2B (y=33.7-3.6x; R^2=1.00)

GAPDH (y=27.3-3.1x; R^2=1.00)

Log [ng Fb RNA]

-0.50 0.00 0.50 1.00

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Figure 6
Floyd et al.

A  GAPDH mRNA
Equivalent
'ng Forebrain RNA'

Control  Chronic EtOH

B  NR1 mRNA
Relative Expression

Control  Chronic EtOH

C  NR2B mRNA
Relative Expression

Control  Chronic EtOH
Figure 7
Floyd et al.

A

Cell A
GAD- Control Neurons
NMDA/Gly + ifenprodil
NMDA/Gly

2A 2B 2C 2D

Cell B
GAD- Control Neurons
NMDA/Gly + ifenprodil
NMDA/Gly

2A 2B 2C 2D

B

Cell C
GAD CE Neurons
NMDA/Gly + ifenprodil
NMDA/Gly

2A 2B 2C 2D

Cell D
GAD CE Neurons
NMDA/Gly + ifenprodil
NMDA/Gly

2A 2B 2C 2D

C

Control Diet

D

EtOH Diet

GAD Negative Neurons

Neuron #