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Ethanol self-administration modulation of NMDA receptor subunit and related synaptic protein mRNA expression in prefrontal cortical fields

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Abstract

Background—Functional impairment of the orbital and medial prefrontal cortex underlies deficits in executive control that characterize addictive disorders, including alcohol addiction. Previous studies indicate that alcohol alters glutamate neurotransmission and one substrate of these effects may be through the reconfiguration of the subunits constituting ionotropic glutamate receptor (iGluR) complexes. Glutamatergic transmission is integral to cortico-cortical and cortico-subcortical communication, and alcohol-induced changes in the abundance of the receptor subunits and/or their splice variants may result in critical functional impairments of prefrontal cortex in the alcohol-addicted state.

Methods and results—The effects of chronic ethanol self-administration on glutamate receptor ionotropic NMDA (GRIN), as well as GRIN1 splice variant mRNA expression was studied in the orbitofrontal cortex (OFC; Area 13), dorsolateral prefrontal cortex (DLPFC; Area 46) and anterior cingulate cortex (ACC; Area 24) of male cynomolgus monkeys. Chronic ethanol self-administration resulted in significant changes in the expression of NMDA subunit mRNA expression in the DLPFC and OFC, but not the ACC. In DLPFC, the overall expression of NMDA subunits was significantly decreased in ethanol treated monkeys. Slight but significant changes were observed for synaptic associated protein 102 kD (SAP102) and neuronal nitric oxide synthase (nNOS) mRNAs. In OFC, the NMDAR1 variant GRIN1-1 was reduced while GRIN1-2 was increased. Furthermore, no significant changes in GFAP protein levels were observed in either the DLPFC or OFC.

Conclusion—Results from these studies provide the first demonstration of post-transcriptional regulation of iGluR subunits in the primate brain following long-term ethanol self-administration. Furthermore, changes in these transcripts do not appear to reflect changes in glial activation or

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loss. Further studies examining the expression and cellular localization of subunit proteins and receptor pharmacology would shed more light on the findings reported here.

Keywords

Ethanol; Glutamate; messenger RNA; Prefrontal Cortex; qPCR; Primate

Introduction

Chronic and excessive alcohol consumption can lead to changes in neuronal structure and function in several brain regions, including the frontal cortex. Previous studies in post-mortem tissue from alcoholics have reported decreased neuronal density in superior prefrontal cortex (Harper and Kril 1991) and cell soma atrophy in the superior frontal and anterior cingulate cortices (Harper and Kril 1989) that is accompanied by decreased dendritic arborization (Harper and Corbett 1990) and loss of large pyramidal neurons in these regions (Harper and Kril 1989). Neuroimaging studies in alcoholics have reported decreased gray and white matter volumes (Jernigan et al. 1991; Pfefferbaum et al. 1997; Pfefferbaum et al. 1998), decreased regional cerebral blood flow (Dally et al. 1988; Melgaard et al. 1990) as well as reduced glucose metabolism in the frontal cortex (Adams et al. 1993; Dao-Castellana et al. 1998).

These structural and metabolic changes are associated with deficits in selective attention, working memory, behavioral inhibition and attribution of stimulus salience (Thorpe et al. 1983; Volkow et al. 1996; Volkow et al. 1999; Elliott et al. 2000b; Elliott et al. 2000a; Goldstein and Volkow 2002; Goldstein et al. 2002). All of these functions are mediated by prefrontal cortex, with attention and working memory more associated with dorsolateral areas and behavioral inhibition more associated with ventral and medial fields. Based on this, we sampled individual fields from each of theses regions: area 46 from dorsolateral cortex, area 13 from orbital cortex and area 24a from medial cortex.

The findings described above were from human subjects with many decades of alcohol exposure and who lived in highly variable environments. It is not clear if the changes described above are due simply to alcohol or whether more subtle changes take place that are due just to alcohol and which may appear long before overt neuropathology is present. Because studies in rodents indicate that alcohol alters glutamatergic synaptic transmission and studies in rodents and humans suggest that the alterations in symaptic transmission are due at least in part to subunit reorganization and expression (Bruckner et al. 1997; Pickering et al. 2007; Raeder et al. 2008; Ridge et al. 2008), we sought to examine this systematically in our model of chronic ethanol self-administration.

Glutamate is the excitatory transmitter responsible for most communication within the nervous system. Even subtle alterations in glutamate-related signaling might therefore disrupt prefrontal cortical information processing. Glutamate signaling is mediated by both metabotropic and ionotropic receptors with the latter implicated in synaptic strength and maintenance as well as a variety of cellular activities including neuronal development, learning and memory, and the reinforcing and neuropathological effects of abused substances (Kalivas et al. 2003; Kalivas et al. 2005).

Ionotropic glutamate receptors (iGluRs) are divided into three classes based on pharmacology and subunit composition – NMDA, AMPA and kainate – each consisting of a combination of four subunits to form a functional ionophore. NMDA receptors require the expression of NR1 in combination with one or more NR2 (A-D) or NR3 (A-B) subunits. The NR1 subunit exists as one of eight splice variants distinguished by the inclusion or

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exclusion of a single N-terminal cassette (N1, exon 5) and/or two C-terminal cassettes, exons 21 and 22 (C1, C2 respectively). Additional biochemical diversity is provided by the existence of splice variants of the NMDAR1 subunit that confer different pharmacological and biochemical properties on the receptor, affect intracellular signaling (Hollmann et al. 1993; Traynelis et al. 1995; Koltchine et al. 1996; Dingledine et al. 1999) and affect receptor subunit trafficking (Horak and Wenthold 2009). These splice variants have been shown to be differentially regulated in various brain regions and during different stages of development, cellular activity, drug treatment and disease processes (Meshul et al. 1996; Le Corre et al. 2000; Loftis and Janowsky 2002; Guilarte and McGlothan 2003; Nagy et al. 2003; Hynd et al. 2004).

In addition to receptor subunit and NR1 splice variant expression, changes in subcellular localization and trafficking of subunits have been shown to contribute to alterations in receptor function (Kumar et al. 2003; Kumar et al. 2004). NMDA receptor localization and association with scaffolding proteins, such as those of the membrane-associated guanylate kinase (MAGUK) family including post-synaptic density 95 (PSD-95) and synaptic associated protein 102 (SAP102), play important roles in the control of downstream signals resulting from NMDA receptor activation (Elias and Nicoll 2007; Lau and Zukin 2007) and may well be affected by ethanol.

Based on available data, we hypothesized that chronic ethanol self-administration would induce differential patterns of expression of NMDA receptor subunit transcripts and NR1 splice variants indifferent prefrontal cortical fields. Therefore, we examined the mRNA expression of NMDA subunits, six NR1 splice variants as well as synaptic proteins, PSD-95, SAP102 and nNOS, associated with the NMDA receptor complexes.

Results

Ethanol self-administration

Individual drinking patterns of the six monkeys included in this study have been reported previously (Monti et al. 2004; Hemby et al. 2006; Anderson et al. 2007). As previously reported, the average daily intakes for 12 months of self-administration ranged from 1.0 -3.33 g/kg ethanol and the average blood ethanol concentrations ranged from 68-188 g/kg (Anderson et al., 2007). Mean daily intake, total intake and blood ethanol concentrations are provided in Table 1. Estimations of these intakes in terms of human drink-equivalents is roughly 0.25 g/kg per drink, or a range of 6-15 drinks/day during the last 6 months of self-administration prior to the brain being harvested. These levels of drinking are considered moderately heavy to heavy in terms of human ethanol consumption (Vivian et al. 2001; Grant et al. 2008).

NMDA subunit mRNA expression

DLPFC (Area 46)—In the DLPFC, we observed a significant difference in NMDA receptor subunit mRNA (GRIN 1, GRIN 2A, GRIN 2B) expression between the groups [F(1,29)=11.486, P=0.002]; however, there was no significant Group x Subunit interaction [F(2,29)=0.688; P=0.512] (Figure 1A). Analysis of GRIN1 variants revealed a trend towards a significant Group x Variant interaction [F(5,59)=2.306; P=0.059] with *post hoc* analysis revealing a significant decrease in variant GRIN1-1 in the ethanol group (Figure 1B). Analysis of synaptic associated protein mRNAs in the DLPFC revealed increased nNOS [F91,9)=16.435, P=0.004) and decreased SAP102 [F(1,9)=8.234, P=0.021) as well as a trend towards decreased PSD 95 [F(1,9)=5.127, P=0.053) in the ethanol group (Figure 2). Correlational analysis revealed significant positive correlations between GRIN1A mRNA levels and average daily intake (r^2=0.819, P=0.0462) and between PDS95 mRNA levels and

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average daily intake (r^2=0.955, P=0.00294) and average blood ethanol concentrations (r^2=0.913, P=0.0109) (Table 2).

OFC (Areas 13a and 13m)—In the OFC, there was no significant difference in NMDA receptor subunit mRNAs between the Groups [F(1,29)=0.556, P=0.463] and no significant Group x Subunit interaction [F(2,29)=2.657, P=0.082] (Figure 3A). In contrast, there was a significant interaction between Group and GRIN variants, due to decreased GRIN 1-1 (P=0.035) and increased in GRIN 1-2 (P=0.001) in the ethanol group (Figure 3B). No significant changes in any of the synapse-associated proteins investigated were observed between the ethanol and control groups (Figure 4).Correlational analysis revealed a significant positive correlation between GRIN2B mRNA levels and total ethanol intake (r^2=0.896, P=0.0158), but not for any of the other transcripts for this region (Table 2).

ACC (Areas 24a and24b)—In contrast to the other prefrontal regions, no significant Main Effect or interactions were observed for any of the GRIN subunits tested (Figure 5 A and B). No significant differences were detected between ethanol and control groups with regard to GRIN1 variant expression in this field. In addition, there were no significant differences in any of the synapse-associated proteins investigated (Figure 6). No significant correlations between transcript levels and average daily intake, total intake or average blood ethanol concentrations were observed (Table 2).

GFAP Western Blot Analysis

We examined GFAP protein levels in cytosolic fractions as a marker of glial cell loss and a measure of possible brain damage that was not visible either in the gross brain specimens or Nissl stained sections. . Ethanol consumption resulted in significantly decreased levels of GFAP in the ACC (t=-7.147, df=8, P<0.01), but not in the DLPFC (t=-0.313, df=8, P=0.762) or OFC (t=-0.581, df=5, P=0.577). For the ACC, there was no significant correlation between GFAP protein levels and daily intake (r^2=-0.026, P=0.961), total intake (r^2=-0.239, P=0.649) or BEC (r^2=-0.107, P=0.840).

Discussion

The current findings are the first demonstration in any primate species that chronic ethanol self-administration can lead to areal specific changes in the expression of iGluR subunit mRNAs in the prefrontal cortex. This provides a possible molecular basis for how glutamatergic transmission is altered by chronic ethanol consumption (Ward et al. 2009). Ethanol self-administration resulted in significant changes in the DLPFC and OFC, but not the ACC – a field-specific distribution of effects like the one we previously reported for changes in $GABA_A$ receptor subunit mRNAs (Hemby et al. 2006).

In DLPFC, NMDA receptor subunit expression was significantly decreased and slight but significant changes were observed for SAP102 and NOS1 mRNA expression as well. In the OFC, the NMDAR1 variant GRIN1-1 was decreased and GRIN1-2 was increased. Because GFAP, a marker of astrocytic activation and glial content was not significantly altered in these fields, it is reasonable to suggest that the observed changes in NMDA receptor subunit expression were not due to neurodegeneration or glial cell loss.A significant decrease in GFAP was detected in the ACC. These data support and expand the results of studies in humans implicating glutamate dysregulation in alcohol abuse (Gass and Olive 2008; Ridge et al. 2008; Schumann et al. 2008; Ridge and Dodd 2009) and provide a potential molecular marker of prefrontal dysregulation in alcoholics (Dao-Castellana et al. 1998; O'Neill et al. 2001; Goldstein et al. 2004).

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Alcohol abuse can be characterized in part by persistent drug-seeking and - taking behaviors that are mediated in part by the DLPFC and OFC. For example, OFC damage leads to deficits in decision-making strategies that depend on the analysis of likely outcome values to arrive at the most profitable outcome (Bechara et al. 1994; Bechara et al. 2000; Pears et al. 2003; Pickens et al. 2005).Alcoholics who who routinely consume large quantities of alcohol despite obvious long-term negative consequences exhibit a similar behavioral deficit (Bechara et al. 2001; Bechara and Damasio 2002; Bechara 2003). The DLPFC, OFC and ACC are commonly reported areas of cue-induced activation by abused substances, including alcohol (Wilson et al. 2004). Previous studies have reported that alcohol craving is associated with increased functional activity of the DLPFC (George et al. 2001; Wrase et al. 2002; Tapert et al. 2004; Olbrich et al. 2006) and OFC (Wrase et al. 2002; Tapert et al. 2003; Myrick et al. 2004).

Monkey are an advantageous model in which to perform these kinds of studies both because their drinking patterns mimic human patters because the monkey prefrontal cortex shares structural similarities with humans that are not present in rodents. In the rat, the PFC is composed only of a set of agranular fields (e.g., dorsal and ventral anterior cingulate, prelimbic and infralimbic fields). Monkey and human brains, by contrast, contain all of these agranular fields as well as dysgranluar and granular fields not seen in the rodent (Carmichael and Price 1994; Ongur and Price 2000).

The mechanisms which mediate the functional dysregulation of prefrontal cortex following alcohol administration are not well understood, but likely involve glutamatergic mechanisms. Studies in rodent cortex indicate either no change or increased NMDA receptor subunit mRNA expression (Morrow et al. 1994; Follesa and Ticku 1995; Snell et al. 1996; Kalluri et al. 1998; Hardy et al. 1999; Pickering et al. 2007; Raeder et al. 2008) depending on route of administration, duration of drinking and time of tissue collection. In humans, NMDA receptor subunit mRNA expression (NR1, 2A and 2B) is decreased in superior frontal and primary motor cortex of cirrhotic alcoholics when subjects were portioned according to the 5HTTLPR allele (Ridge et al. 2008). Because of the neurotoxic nature of chirrhosis, it is not clear what the causes of these changes may have been. Our current report of a significant decrease in NMDA receptor subunit mRNAs as well as decreased NR1-1 variants in the DLPFC and decreased NR1-1 and increased NR1-2 variant mRNA levels in the OFC following chronic ethanol self-administration, suggest that the changes seem in human subjects are indeed due to acohol.

In addition to changes in receptor subunit mRNA expression, other proteins such as PSD95, SAP102 and nNOS interact with NMDA receptors and contribute to synaptic strength and plasticity. PSD-95 and SAP102 are members of the membrane associated guanylate kinase family of proteins, which couple NMDA receptors to signaling complexes that regulate activity-dependent changes in synaptic strength and participate in NMDA receptor-dependent forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (Migaud et al. 1998; Beique and Andrade 2003; Stein et al. 2003; Yao et al. 2004; Beique et al. 2006; Cuthbert et al. 2007). Previous studies have shown that nNOS contributes to NMDA receptor induced neuroplasticity as well as cerebral blood flow changes induced by NMDA receptor activation (Iadecola and Nedergaard 2007; Garthwaite 2008). Nitric oxide (NO) is synthesized via nNOS which is linked to the NR2 subunit of the NMDAR via PSD95 (Brenman and Bredt 1997; Christopherson et al. 1999). The biological relevance of the slight, but statistically significant, increase in nNOS mRNA expression in the DLPFC following chronic alcohol exposure deserves further investigation.

The present findings provide additional important insight into potential mechanisms of prefrontal cortical dysregulation following chronic alcohol use. Previously, using the same

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subjects examined in the present study, we reported regulation of specific GABA-A subunit mRNA expression in the DLPFC and OFC, but not the ACC, (Hemby et al. 2006). Thus, changes in both NMDA and GABA-A receptor configuration in DLPFC and OFC may underlie the decision making deficits resulting from chronic alcohol abuse (Bechara and Damasio 2002; Goldstein and Volkow 2002; Bechara 2003). Examination of the effects of ethanol on protein levels of receptor subunit expression, the class of neurons that are primarily affected, and the expression of related trafficking and anchoring proteins and their genes within these prefrontal regions will be fundamental to ascertaining the molecular pathology of prefrontal dysregulation in alcoholism.

Experimental Procedures

Subjects and ethanol self-administration

Ten adult male cynomolgus monkeys *(Macaca fascicularis*; 5.5 – 6.5 years at beginning of experiment) were subjects for the present experiments (n=6 ethanol group, n=4 control group). The drinking model and the animals behavior has been extensively analyzed elsewhere. Six adult male cynomolgus monkeys were quarantined for 2 months and then transferred to the laboratory primate housing room and assigned a cage within a quadrant rack as described previously. Attached to one wall of each monkey's home cage was an operant panel that allowed access to all fluid and food requirements. Monkeys were trained to operate the drinking panel in daily 60-minute sessions and then induced to drink water and later ethanol (4% w/v in water). Following 120 days of induction, scheduled pellet delivery was discontinued. For 6 months, ethanol and water were available *ad libitum* and food was available in meals during daily 16-hour sessions. Following that, the monkeys underwent ethanol abstinence for 12 months. After the abstinence period, the monkeys were again allowed access to 4% ethanol, water, or food from the panel for 22 h/d for a range of 558 to 595 consecutive days (approximately 18 months). During the *ad libitum* period monkeys were allowed to self-administer 4% (w/v) ethanol daily, with the volume and rate of alcohol consumed determined solely by the monkey. Once the animals are given free access to ethanol, the onset of daily 22-hour sessions are signaled by the illumination of amber stimulus lights above both drinking spouts. Both 4% (w/v) alcohol and water were available at all times during the daily 22-hour sessions. In addition, a "meal structure" was imposed so monkeys were required to eat the daily allotment of food in no less than three "meals," with at least two hours between each meal.

Experimentally naïve control subjects (n=4) were the same as those described previously (Ivester et al. 2007). Following 2 months of quarantine, monkeys were placed on the same diet as the ethanol-drinking animals (Vivian et al. 2002) and had very similar daily routines as the ethanol-drinking animals. Control individuals remained in the laboratory for 6 months prior to euthanasia.

Blood samples (20 μL) for the analysis of blood ethanol concentrations (BEC) were taken from the saphenous vein every fifth day from every monkey just before the lights turning off in the room and approximately seven hours following the onset of the session. Blood samples were sealed in air-tight vials containing 500 μl of distilled water and 20 μL of isopropanol (10%; internal standard) and stored at −4°C until assay using a gas chromatograph (Hewlett-Packard 5890 Series II, Avondale, PA) equipped with a headspace autosampler, flame ionization detector, and a Hewlett Packard 3392A integrator. All behavioral studies were conducted in the laboratory of Dr. Grant at Wake Forest University. The care of the animals and euthanasia procedures in this study were performed according to the National Institutes for Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Wake Forest University.

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Necropsy

Following euthanasia, the frontal cortex was dissected from the rest of the brain and the orbitofrontal cortex (OFC; Area 13), anterior cingulate cortex (ACC; Area 24) and dorsolateral prefrontal cortex (DLPFC; Area 46) were dissected as described previously (Hemby et al. 2006). The OFC dissection included the medial and lateral aspects of the medial orbital sulcus located rostral to the rostral end of the corpus callosum, and therefore included Areas 13a and 13m (Carmichael and Price 1994). The ACC was dissected from the rostral pole of the corpus callosum and included tissue from the cingulate gyrus up to but not including the lower bank of the cingulate sulcus, and therefore contained fields 24a and 24b. The DLPFC (Area 46) was dissected from the banks of the principle sulcus midway along its length.

Messenger RNA abundance

RNA Isolation and cDNA synthesis: Total RNA was isolated using Trizol (Sigma-Aldrich, St. Louis, MO) followed by chloroform extraction/isopropanol precipitation and stored at −80°C. 2 µg of total RNA from each sample, as well as a pool of total RNA combined from the 10 cynomologus monkeys, was reverse transcribed using random primers and SuperScript III kits (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Resulting cDNA product was diluted 1:100 with RNAse-free water for samples, and cDNA from the pooled samples was serially diluted in 2-fold dilutions from 1:20-1:640 for use as standards.

Real-time quantitative PCR: Standard Taqman assays were purchased for measurement of specific NMDA receptor subunit levels (GRIN1: Hs00609557_m1; GRIN2A: Hs00168219_m1; GRIN2B: Hs00168230_m1, PSD95: Hs00176354_m1; SAP102: Hs01020278_m1; nNOS: Hs00167223_m1, Applied Biosystems, Foster City, CA). Custom Taqman assays were designed for six NR1 splice variants in collaboration with Applied Biosystems (Foster City, CA). Two assays distinguish between the N-terminal variants (exon 5), NR1A and NR1B, while four assays distinguish between the C-terminal variants (exons 21 and 22), NR1-1, NR1-2, NR1-3 and NR1-4. All assays used FAM reporter dye and NFQ quencher and were designed such that the Taqman probe spanned the exon-exon junction (or included exon) that is specific to the splice variant being measured. Sequence information for these assays is listed in Table 2.

Using a 384 well format with the ABI Prism 7900HTS real-time detector, 0.5 l aliquots of Taqman Expression Assay (20X), 5.0 l 2X Absolute QPCR ROX PCR Mastermix (Abgene), and 4.5µl diluted cDNA (either sample or pooled standard) were mixed together and pipetted into single wells of the PCR plate. For no template controls (NTC) for each gene tested, water was added in lieu of cDNA. Each sample, including NTC was run in triplicate. Thermocycling conditions: 1) one cycle 2 min at 50°C, 2) one cycle 15 min at 95°C, and 3) 40 cycles 15 sec at 95°C and 1 min at 60°C. Fluorescence was measured during the 60°C step for each cycle. Reactions were quantified by the standard curve method using SDS2.1 software generating a mean quantity value (Qty mean) for each sample from the triplicates of that sample for each gene of interest. Endogenous controls were selected from a set of seven candidate reference transcripts: -actin (ACTB), ribosomal protein 18S (18S), TATA box binding protein (TBP), hypoxanthine phosphoribosyltransferase 1 (HPRT1), peptidylprolyl isomerase A (cyclophilin A) (PPIA), -glucuronidase (GUSB), and phosphoglycerate kinase 1 (PGK1) using geNorm software. geNorm is a collection of VBA macros for Microsoft Excel which allows the determination of the most stable reference genes from a given test panel of genes. By computing the average pairwise variation (V) for each control gene paired with all other tested control genes, geNorm calculates the gene expression stability measure (M) (Vandesompele et al. 2002). This allows for the selection

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of the most stably expressed control genes in a given sample set, minimizing any bias in the data as a result of normalization (see Supplementary Data). The gene expression normalization factor is calculated based on the geometric mean of a user-defined number of reference genes (Vandesompele et al. 2002). Three candidate genes (PGK1, Hs99999906_m1; TBP, Hs99999910_m1; and HPRT, Hs99999906_m1) were selected to serve as endogenous controls. Data for each gene of interest was expressed as Qty mean for the gene of interest/geometric mean of Qty mean values for the selected endogenous control genes. Normalized values are expressed as percent control.

Western Blot analysis—Cytoslic fractions were collected from tissue samples as described previously in (Tang et al. 2004; Hemby et al. 2005a; Hemby et al. 2005b). Protein concentrations were calculated using the bicinochoninic acid protein assay kit (Pierce, Rockford, IL) and diluted in Laemmli sample buffer to achieve the equivalent final protein concentrations. Sample buffer is added to 10 μg of protein from each sample and heated to 95°C for 5 minutes. Proteins were separated on pre-cast 10% Tris-HCl SDS-PAGE gels (Biorad) and transferred to nitrocellulose membranes. Membranes were blocked an incubated with the anti-GFAP antibody (1:5000; Synaptic Systems; Goettingen, Germany) overnight in Odyssey blocking buffer (Licor, Lincoln, NE). Visualization was accomplished with AlexFluor680 and IRDye800 labeled secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA and Molecular Probes, Eugene, OR). Blots were scanned with a Licor Odyssey infrared scanner and signals quantified with Odyssey version 1.2 software. Equal protein loading and efficiency of transfer were confirmed by probing blots with an anti-neuronal tubulin antibody (1:15,000; Millipore, Billerica, MA). Background-subtracted intensity values for each sample were calculated.

Currently available NR1 splice variant antibodies will detect the N1, C1, C2 and C2′ cassettes, respectively, but will not differentiate splice variants NR1-1 from NR1-2 (both contain the C2 cassette), NR1-1 from NR1-3 (both contain the C1 cassette) or NR1-3 from NR1-4 (both contain the C2′ cassette) and therefore would not enable correlative analysis of protein levels for the current study.

Statistical Analysis: Levels of gene expression were calculated using SDS 2.1 software (Applied Biosystems, Foster City, CA) to interpolate the Ct values for each well onto a standard curve generated from the Ct values of a dilution series of standards. These quantity values were then averaged across triplicates after removal of outliers and expressed relative to the quantity value for the mean of the endogenous control genes measured in the same sample on the same plate. This relative value (gene of interest Qty mean/endogenous control Qty mean) was used for subsequent statistical analysis. Experiments determining relative gene expression for each candidate gene were run independently. For each gene, two-way analysis of variance was employed with Group (Ethanol and Control) and Subunit (per receptor class) as the Main factors and mRNA abundance as the dependent variable. All subunits for a receptor class were analyzed for each brain regions separately. Bonferroni's test was used for post hoc analyses. For Western blot analysis of GFAP, data were analyzed using a one way ANOVA with hybridization intensity as the dependent variable. Post-hoc analysis was performed using Tukey's test. Null hypotheses were rejected when $P<0.05$.

Supplementary Material

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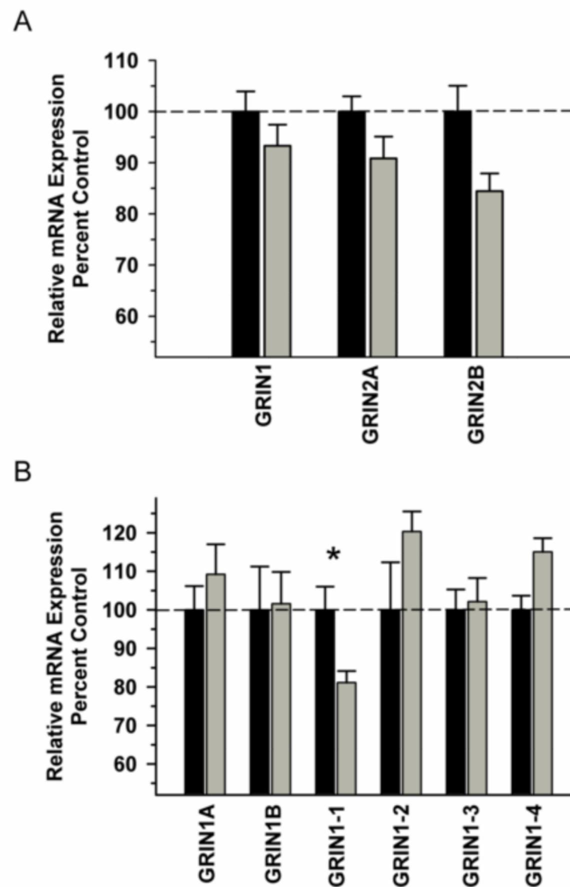

Figure 1.

Effect of chronic self-administration by cynomolgus macaques on NMDA subunit expression in DLPFC. Ethanol induced a significant decrease in NMDA receptor subunit expression; however, there was no Group x Subunit interaction (A). A trend towards a significant interaction was observed for GRIN1 splice variant expression, with *post hoc* analysis revealing a significant decrease GRIN1-1 in the ethanol group (B). Black bar – control subjects, gray bar – ethanol subjects. Asterisks indicate a significant difference compared to control subjects ($P<0.05$).

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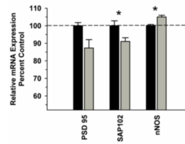

Figure 2.

Effect of chronic self-administration on PSD-95, SAP102 and nNOS mRNA expression in the DLPFC. Ethanol induced a significant decrease in SAP102 mRNA, a significant increase in nNOS mRNA and a slight decrease in PSD95 mRNA levels (P=0.053). Black bar – control subjects, gray bar – ethanol subjects. Asterisks indicate a significant difference compared to control subjects ($P<0.05$).

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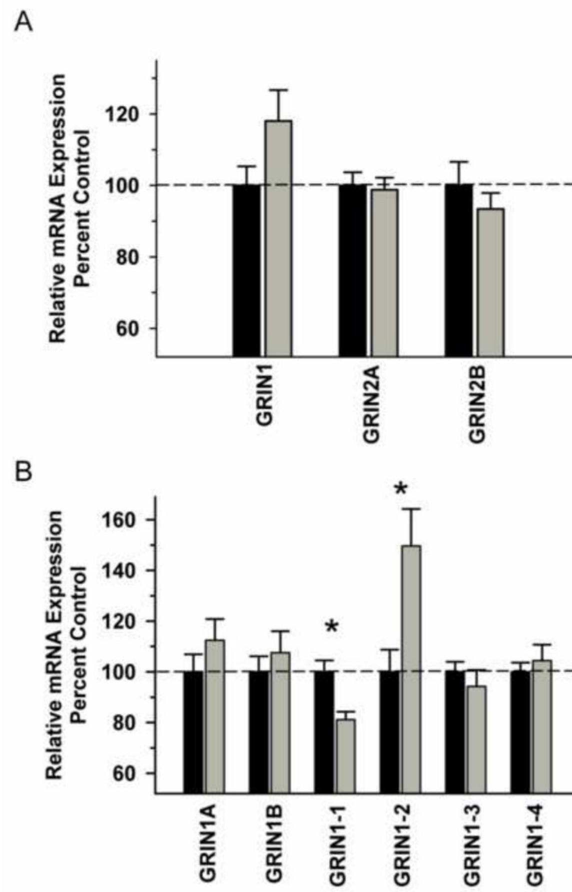

Figure 3.

Effect of chronic self-administration by cynomolgus macaques on NMDA subunit expression in OFC. Ethanol self-administration did not affect NMDA receptor subunit mRNA expression in this region (A). A significant interaction was observed for GRIN1 splice variant expression, with *post hoc* analysis revealing a significantly decreased GRIN1-1 expression and increased GRIN1-2 expression in the ethanol group (B). Black bar – control subjects, gray bar – ethanol subjects. Asterisks indicate a significant difference compared to control subjects ($P<0.05$).

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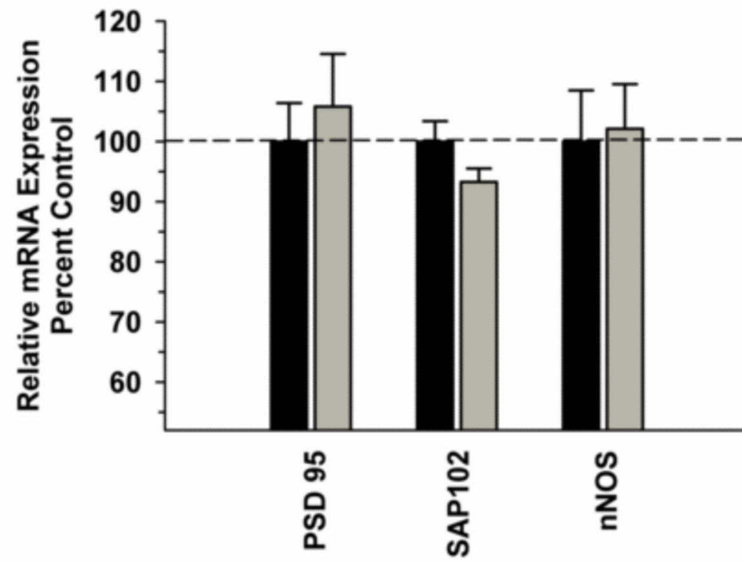

Figure 4.

Effect of chronic self-administration on PSD-95, SAP102 and nNOS mRNA expression in the OFC. No significant differences were observed between the groups for PSD-95, SAP102 and nNOS expression. Black bar – control subjects, gray bar – ethanol subjects.

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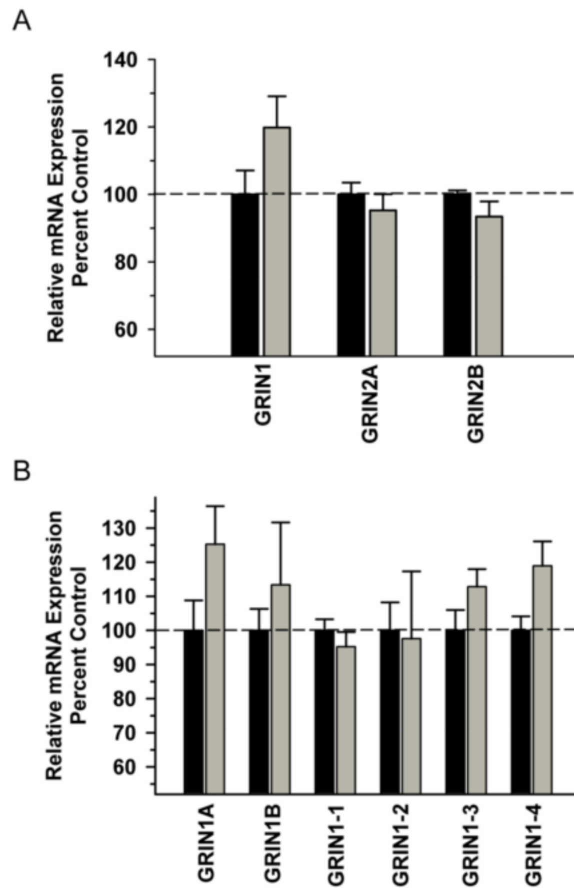

Figure 5.
Effect of chronic self-administration by cynomolgus macaques on NMDA subunit expression in ACC. Ethanol induced a significant decrease in NMDA receptor subunit expression; however, there was no Group x Subunit interaction (A). A trend towards a significant interaction was observed for GRIN1 splice variant expression, with *post hoc* analysis revealing a significant decrease GRIN1-1 in the ethanol group (B). Black bar – control subjects, gray bar – ethanol subjects. Asterisks indicate a significant difference compared to control subjects ($P<0.05$).

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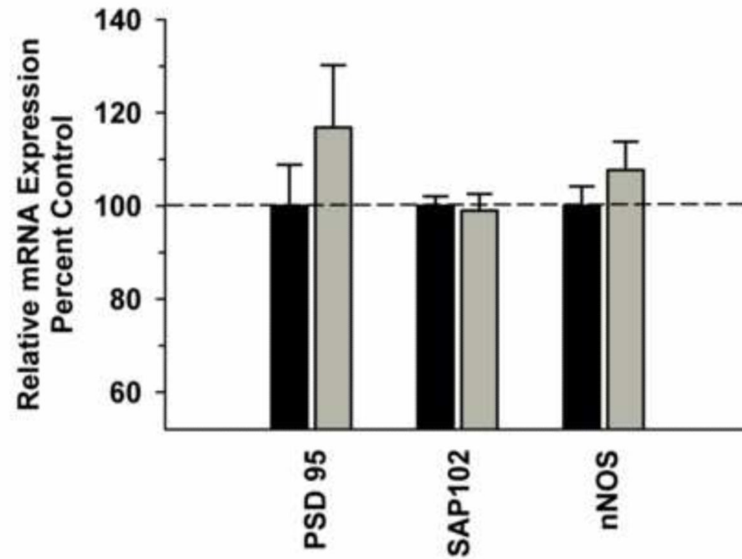

Figure 6.

Effect of chronic self-administration on PSD-95, SAP102 and nNOS mRNA expression in the ACC. Ethanol induced a significant decrease in PSD-95 and SAP102 mRNAs and a slight increase in nNOS expression. Black bar – control subjects, gray bar – alcohol subjects. Asterisks indicate a significant difference compared to control subjects ($P<0.05$).

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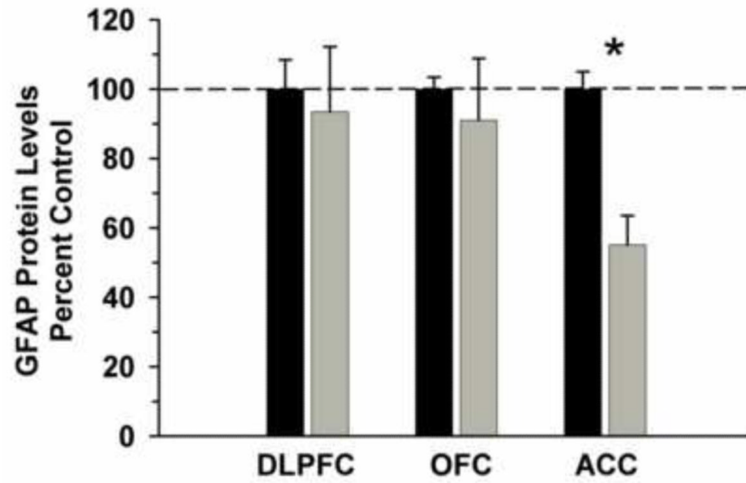

Figure 7.
GFAP protein levels in prefrontal cortical brain regions of ethanol consuming and control cynomolgus monkeys. Cytosolic fractions were isolated and separated on 10% SDS-PAGE. Data are expressed as mean (± S.E.M.) of the percent of control values per amount of protein loaded. Asterisks indicate a significant difference ($P<0.05$).

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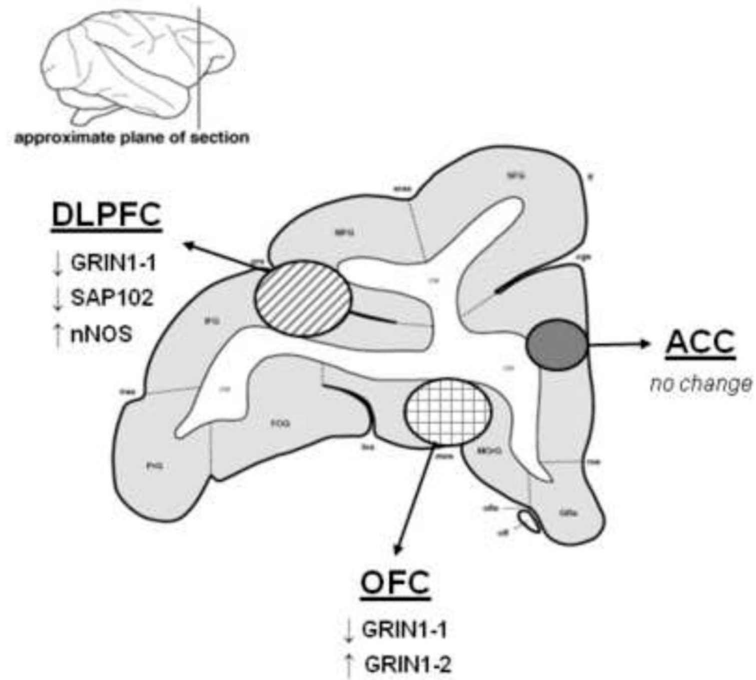

Figure 8.
Summary of changes in NMDA receptor subunit and NMDAR1 variants in vulnerable prefrontal cortical regions following ethanol consumption in cynomolgus monkeys.

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Table 1
Ethanol Consumption for monkeys used in this study

Daily intake values and blood ethanol concentrations represent mean ± S.E.M.

Subject	Daily Intake (g/kg)	Total Intake (g/kg)	Blood Ethanol Concentration (n)
5404	3.33 ± 0.24	2289.8	188 ± 17 (33)
5497	1.76 ± 0.10	1670.8	105 ± 11 (33)
6101	1.85 ± .012	1524.2	100 ± 9 (28)
6304	1.17 ± 0.13	897.8	63 ± 13 (30)
6305	1.49 ± 0.08	1184.5	62 ± 10 (30)
6306	2.70 ± 0.17	2757.7	173 ± 10 (35)

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Table 2

Correlational analysis of gene expression with average daily intake, total intake and average blood ethanol concentrations

Values expressed are Pearson correlation coefficients (r^2) for the six male monkeys that consumed alcohol in the present study.

	DLPFC			OFC			ACC		
	Daily Intake	Total Intake	BEC	Daily Intake	Total Intake	BEC	Daily Intake	Total Intake	BEC
GRIN1	0.381	0.539	0.376	0.288	0.402	0.222	0.326	0.451	0.405
GRIN2A	0.321	−0.0166	0.120	0.680	0.596	0.587	0.654	0.521	0.544
GRIN2B	0.471	0.586	0.473	0.757	**0.896** *	0.768	0.528	0.742	0.551
GRIN1-1	0.675	0.292	0.587	0.558	0.595	0.609	−0.0258	−0.0652	−0.119
GRIN1-2	0.112	0.101	0.00863	0.479	0.446	0.458	0.633	0.244	0.502
GRIN1-3	0.0731	−0.193	0.0216	0.278	−0.0216	0.232	0.132	−0.219	−0.0453
GRIN1-4	−0.250	−0.271	−0.209	0.263	−0.0647	0.240	0.737	0.384	0.643
GRIN1A	**0.819** *	0.595	0.770	0.663	0.642	0.588	0.459	0.420	0.447
GRIN1B	−0.256	−0.0326	−0.248	0.0817	0.198	0.0337	0.511	0.368	0.437
PSD95	**0.955** **	0.765	**0.913** *	0.422	0.704	0.455	0.462	0.491	0.497
SAP102	0.548	0.526	0.452	0.717	0.633	0.633	0.240	0.298	0.208
nNOS	0.142	−0.180	−0.0649	0.194	0.313	0.111	0.523	0.490	0.582

* $P<0.05$

** $P<0.01$

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Table 3

Sequence information for custom-designed NR1 splice variant TaqMan assays

Gene	Probe Sequence	Forward Primer	Reverse Primer	Size
NR1a	TCTGCCTTGGACTCAGG	CGACGACCACGAGGGC	GACCCGGGCCTCCAG	151
NR1b	AACCTCGACCAACTGTCC	TCAGCGACGACCACGAG	GCACCTTCTCTGCCTTGGG	147
NR1-1	CCGGTGCTCGTGTCTT	CGGAAGAACCTGCAGGATAGAAAG	CGGCAGCACTGTGTCTTTT	180
NR1-2	CCGGTGCTCTGCAGGTT	AGCGGCACAAGGATGCT	GGCCCTCCTCCCTCTCAATAG	159
NR1-3	ATGGTACTGCGTGTCTTT	GAAGAACCTGCAGGATAGAAAGAGT	TGCTGACCGAGGGATCTGA	182
NR1-4	ACTGCTGCAGGTTCTT	GGCCGGGATCTTCCTGATTT	GCTGACCGAGGGATCTGAGA	172