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Chronic Ethanol Consumption Alters Glucocorticoid Receptor Isoform Expression in Stress Neurocircuits and Mesocorticolimbic Brain Regions of Alcohol-Preferring Rats

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Abstract

Evidence suggests the hypothalamic–pituitary–adrenal (HPA) axis is involved in Alcohol Use Disorders (AUDs), which might be mediated by an imbalance of glucocorticoid receptor (GR), GRα and GRβ, activity. GRβ antagonizes the GRα isoform to cause glucocorticoid (GC) resistance. In the present study, we aimed to investigate the effects of chronic continuous free-choice access to ethanol on GR isoform expression in subregions of the mesocorticolimbic reward circuit. Adult male alcohol-preferring (P) rats had concurrent access to 15% and 30% ethanol solutions, with *ad lib* access to lab chow and water, for six weeks. Quantitative Real-time PCR (RT-PCR) analysis showed that chronic ethanol consumption reduced GRα expression in the nucleus accumbens shell (NAcsh) and hippocampus, whereas ethanol drinking reduced GRβ in the nucleus accumbens core (NAcc), prefrontal cortex (PFC), and hippocampus. An inhibitor of GRα, microRNA-124–3p (miR124–3p) was significantly higher in the NAcsh, and GC-induced gene, GILZ, as a measure of GC-responsiveness, was significantly lower. These were not changed in the NAcc. Likewise, genes associated with HPA axis activity were not significantly changed by ethanol drinking [i.e., corticotrophin-releasing hormone (*Crh*), adrenocorticotrophic hormone (*Acth*), and proopiomelanocortin (*Pomc*)] in these brain regions. Serum corticosterone levels were not changed by ethanol drinking. These data indicate that the expression of GRα and GRβ isoforms are differentially affected by ethanol drinking despite HPA-associated peptides remaining unchanged, at least at the time of tissue harvesting. Moreover, the results suggest that GR changes may stem from ethanol-induced GC-resistance in the NAcsh. These findings confirm a role for stress in high ethanol drinking, with GRα and GRβ implicated as targets for the treatment of AUDs.

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Keywords

GRα; GRβ; P rats; nucleus accumbens; ethanol

INTRODUCTION

Studies on the glucocorticoid system have shown its dysregulation during ethanol intoxication and withdrawal as well as its involvement in the development of alcohol (ethanol) use disorders (AUDs). For example, mifepristone, an antagonist at glucocorticoid receptors (GRs), can reduce ethanol seeking and drinking behaviors (Vendruscolo et al., 2012). The Hypothalamic Pituitary Adrenal (HPA) axis activity produces adrenocorticotrophic hormone (ACTH) that induces the release of the stress hormone cortisol (corticosterone in rodents, a primary glucocorticoid, GC) from the adrenal glands (Kadmiel and Cidlowski, 2013), which can be dose-dependently suppressed by acute ethanol exposure (Lee and Rivier, 1994). Hence, short-term lower dose ethanol exposure can reduce objective stress measures. However, long-term chronic alcohol exposure has the opposite effect and increases GC levels, and by extension is associated with ethanol dependence and withdrawal (Sun et al., 2013). One possible link is the ACTH precursor polypeptide proopiomelanocortin (*POMC*) gene, which is primarily expressed in the arcuate nucleus of the hypothalamus, the nucleus of the solitary tract, as well as the pituitary gland (Niikura et al., 2013). Zhou et al. showed that female and male conditional (restricted to the hypothalamus) *Pomc* knock out (KO) mice displayed reduced ethanol drinking and preference behaviors (Zhou et al., 2017), thus confirming a role for HPA axis activity in AUDs.

However, the interaction of these stress factors is complex, with GCs able to reduce POMC, ACTH, as well as corticotrophin releasing hormone (CRH) expression and by extension HPA activity (Kadmiel and Cidlowski, 2013). Early research showed that chronic ethanol drinking can interfere with HPA activity and cortisol responsiveness (Adinoff et al., 1990; Richardson et al., 2008). This, in turn, can lead to sensitization of extrahypothalamic stress systems, e.g., amygdaloid CRH activity (Makino et al., 2002). The role of HPA activity has been extensively studied in excessive drug, including ethanol, taking behavior [for review see (Wand, 2008)]. For instance, ethanol dependence and withdrawal are associated with the dysregulation of HPA activity, including elevated serum GC concentrations and inhibition of the HPA response to subsequent stressors (Wand and Dobs, 1991; Lee et al., 2000). One hypothesis is that overstimulation by GCs plays a critical role in the development of ethanol dependence and withdrawal. However, little research has explicitly measured the responsiveness and expression levels of GR isoforms in subregions of the mesocorticolimbic reward circuit following chronic ethanol intake.

Two primary isoforms of hGR have been identified (GRα and GRβ), which are the result of alternative splicing of exon 9 (exon 9α or 9β) (Hollenberg et al., 1985). In rodents, the GRβ was identified as a result of alternative splicing of intron 8 (Hinds et al., 2010; DuBois et al., 2013), which inhibits GRα activity (Hinds et al., 2010). The GRα is the classic GR that principally mediates GC responsiveness *in vivo* (Rivers et al., 2009; Oakley and Cidlowski,

2013). The GRβ lacks helix 12 of the ligand-binding domain (LBD), which interferes with the ability to bind GCs although it remains transcriptionally active (Stechschulte et al., 2014; Hinds et al., 2016; John et al., 2016; Marino et al., 2016; McBeth et al., 2016). GRβ influences GC-mediated gene transcription by the formation of GRα-GRβ heterodimers, and it is considered an inhibitor of GRα by recruiting his-tone deacetylases (HDACs) (Oakley et al., 1999; Oakley and Cidlowski, 2011). It has been shown that increasing the expression of GRβ causes immunological disorders associated with GC-resistance, including GC-resistant asthma which can be fatal (Longui et al., 2000; Goecke and Guerrero, 2006; Zhang et al., 2007). Regarding immune function, GRβ promotes astrocyte proliferation and migration following injury (Yin et al., 2013) as well as bladder cancer migration (McBeth et al., 2015). Furthermore, the state of GC sensitivity and resistance may be determined by the ratio of the GRα/GRβ isoforms, such that a low ratio could be indicative of GC-resistance (Lewis-Tuffin and Cidlowski, 2006). Although both GRα and GRβ are co-expressed in brain regions, their relative abundance in reward neurocircuitry remains largely unknown. In addition, the role of GR function in AUDs and substance use disorders (SUDs), including regulation of HPA activity, has not been established.

Studies have demonstrated that treatment with the ‘traditional’ GR antagonist mifepristone reduced ethanol intake in rats using a limited access procedure (Koenig and Olive, 2004). In addition, mifepristone attenuated compulsive-like ethanol intake in alcohol-dependent Wistar rats and decreased excessive ethanol drinking and craving in alcoholics (Vendruscolo et al., 2015). Furthermore, mifepristone reduced yohimbine-induced reinstatement of ethanol-seeking in male Long-Evans rats (Simms et al., 2012). While one could argue that mifepristone inhibition of GRα is mediating these effects, it also binds to other nuclear receptor transcription factors, such as the progesterone receptor (Ponikwicka-Tyszko et al., 2019). Regarding HPA activity and amygdaloid activity, CRH antagonists have been shown to reduce ethanol self-administration in alcohol-dependent male Wistar rats (Roberto et al., 2010). Evidence has also revealed an association between DNA methylation of the *POMC* gene promoter region and ethanol craving (Muschler et al., 2010).

GR function may change in the extrahypothalamic stress system during ethanol dependence (Vendruscolo et al., 2012), such that acute ethanol withdrawal downregulated ‘total’ GR mRNA in the prefrontal cortex (PFC), nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST) of alcohol-dependent Wistar rats. Additionally, protracted ethanol abstinence has been associated with upregulation of ‘total’ GR mRNA levels in the NAc-shell, ventral BNST, and central nucleus of the amygdala (CeA), which mediate relapse-like behaviors (Vendruscolo et al., 2012). However, the expression of individual GRα and GRβ isoforms was not tested in these studies. Given the importance of GR isoforms and their inverse functionality, herein we investigated the effect of chronic ethanol consumption on the expression of GRα and GRβ isoforms, GRα suppressor miR124–3p (Ledderose et al., 2012), GC-controlled gene glucocorticoid-induced leucine zipper (*Tsc22d3*, commonly referred to as GILZ) (Hinds et al., 2011; Hinds et al., 2014), as well as serum corticosterone. In addition, we examined expression levels of HPA activity regulators CRH and POMC in mesocorticolimbic subregions in alcohol-preferring (P) rats given chronic free-choice access to ethanol.

EXPERIMENTAL PROCEDURES

Animals

Male P rats were individually housed in wood-chip-bedded plastic cages at Indiana University School of Medicine (Indianapolis, IN, USA), and were acclimated in a 21 °C temperature and 50% humidity room with a 12/12 h light/dark cycle. Rats had access to food and water *ad libitum* throughout the experimental procedures. All treatment procedures were performed under Dr. Richard L. Bell's protocol that was approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine (Indianapolis, IN, USA) in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences).

Ethanol-drinking procedure

Adult male alcohol-preferring (P) rats ($n = 16$) were used herein. The P rats ($n = 8$) were given continuous free-choice access to 15% and 30% ethanol (v/v, available concurrently) for six weeks. There was also an ethanolnaïve group ($n = 8$). All rats had *ad lib* access to food and water. Ethanol consumption was measured daily as grams of ethanol consumed per kilogram of rat body weight per day (g/kg/day) by subtracting the weight of the bottle from its previous weight. P rats that consumed 4 g/kg/day and less were excluded from the study since they did not meet the criterion of development of ethanol dependence as it was established in previous studies from our laboratory (Sari et al., 2011).

Brain tissue harvesting

At the end of week 6, P rats were euthanized by CO2 inhalation, rapidly decapitated with a guillotine, with their brains removed and stored at −80°C. The infralimbic prefrontal cortex (IL-PFC), prelimbic prefrontal cortex (PL-PFC), nucleus accumbens core (NAc-core), and shell (NAc-shell), hippocampus, amygdala, hypothalamus, and pituitary gland were micropunched stereotaxically using a cryostat apparatus maintained at −20 °C. We have followed the stereotaxic coordinates for the rat brain (Paxinos et al., 2007) to isolate the brain regions of interest following visualized landmarks. The brain regions then were frozen at −80 °C for further experiments.

RNA extraction of mRNA or microRNA for real-time PCR analysis

Total RNA was extracted from tissue lysate using Trizol (Invitrogen) and RNeasy mini kit (Qiagen) according to the manufactures' protocol. Tissues were homogenized in Trizol buffer and 3.5 volumes of 100% ethanol were added to the samples. The RNeasy mini spin column was used to extract the total RNA. Total RNA was quantitated on Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and cDNA for mRNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or miScript II RT Kit (Qiagen) was used for miRNA cDNA synthesis. PCR amplification of the cDNA was performed by quantitative real-time PCR using TrueAmp SYBR Green qPCR SuperMix (Smart Bioscience). The thermocycling protocol consisted of

10 min at 95 °C, 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C, and 20 sec at 72 °C and completed with a melting curve ranging from 60 to 95 °C to allow distinction of specific products. A reaction with primers to GRα and GRβ isoforms, GILZ, CRH, and POMC, with a separate reaction with primers to GAPDH for normalization, was performed. PCR amplification of the miRNA-cDNA was performed by quantitative real-time PCR using miScript SYBR Green PCR Kit (Qiagen). The thermocycling protocol consisted of 15 min at 95 °C, 40 cycles of 15 sec at 94 °C, 30 sec at 55 °C, and 30 sec at 70 °C and finished with a melting curve ranging from 60 to 95 °C to allow distinction of specific products. The miScript Primer Assay primers were purchased from Qiagen. Normalization was performed in separate reactions with primers to Hs_RNU6–2_11, At_ U19_1, and Hs_SNORD61_11.

Corticosterone measurement

Corticosterone levels were measured via a Corticosterone ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA). Samples were extracted using methylene chloride and solvent was evaporated. A 1:500 dilution of each reconstituted sample was made with the extraction buffer for further analysis. Controls, standard curve, and sample for each condition ($n = 8$) was loaded in duplicate or triplicate into a 96-well plate and incubated overnight at 4 °C. Plate was washed 3 times and wells were developed in the dark for 2 hours. Plate was read at 412 nm on the Versamax Microplate Reader (Molecular Devices, San Jose, CA, USA).

Statistical analysis

Data were analyzed with Prism 5 (GraphPad Software, San Diego, CA, USA) using two tailed, unpaired *t*-test. Samples of ethanol-exposed group and water-control group had a normal distribution using D'Agostino-Pearson normality test ($p > 0.05$) and F test to compare the variances (p values range from less than 0.001 to 0.9187). The F test results may be ignored with equal, or nearly equal, and moderately large sample size (Moser and Stevens, 1992; Hayes et al., 2007). Results are expressed as mean ± SEM. p values of 0.05 or smaller were considered statistically significant. Outliers were excluded using GraphPad QuickCalcs: outlier calculator.

RESULTS

Effect of chronic ethanol consumption on the serum corticosterone level

P rats were given free access to water or 15% and 30%, the latter available concurrently, ethanol for six weeks (Fig. 1A). Fig. 1B shows the mean (±SEM) of total ethanol intake in P rats during the 6 weeks of drinking. The average ethanol intake achieved for the 6th week was 6.87 g/kg/day. P rats maintained average drinking above ~5 g/kg/day, which is essential to meet the criterion of developing of ethanol dependence (Li et al., 1987). We examined the effect of chronic ethanol drinking on the serum corticosterone level. Results showed that chronic ethanol drinking did not significantly change the levels of serum corticosterone (Fig. 1C).

Effects of chronic ethanol consumption on GRα and GRβ expression in the NAc-core and -shell

We first measured the effect of chronic ethanol consumption on the gene expression of the GR isoforms in the NAc-core and NAc-shell (Fig. 2A, D). Chronic ethanol consumption significantly reduced the gene expression of GRβ in the NAc-core ($t(12) = 2.993$, $p \leq 0.05$), while the GRα gene expression was not altered. There was no significant change in total GR (*Nr3c1*) expression between the ethanol-exposed group and the water-control group. Higher GRα/GRβ ratio indicates significantly altered GC-sensitivity in the NAc-core ethanol-exposed group ($p < 0.05$) (Fig. 2A, right). However, both GRα ($t(13) = 4.39$, $p \leq 0.001$) and total GR (*Nr3c1*) ($t(13) = 3.23$, $p \leq 0.01$) expression were significantly downregulated in the NAc-shell in the ethanol-exposed group compared to the water-control group ($n = 7–8$), whereas GRβ expression remained unchanged. The GRα suppressor, microRNA-124–3p (miR124–3p), was significantly higher in the NAc-shell (Fig. 2E), but not the NAc-core (Fig. 2B). Thus, the GRα/GRβ ratio was not changed significantly in the NAc-shell (Fig. 2D), despite significant changes in the NAc-core. This suggests that GC-insensitivity may occur in the NAc-shell due to miR124–3p suppression of GRα.

Effects of chronic ethanol consumption on the expression of total GR, GRα, and GRβ in the hippocampus.

The total GR expression was significantly downregulated in the hippocampus of ethanol-exposed group compared with the water-control group ($n = 7–8$) (Fig. 4A-right, $t(13) = 2.191$, $p \leq 0.05$). Which parallels the finding that chronic ethanol consumption significantly decreased the expression of GRα ($t(13) = 4.374$, $p \leq 0.001$) and GRβ ($t(13) = 4.638$, $p \leq 0.001$) isoforms. Because both isoforms were reduced, the GRα/GRβ ratio was not changed between ethanol-exposed and water-control rats (Fig. 4A-left).

Effects of chronic ethanol exposure on the expression of the total GR, GRα and GRβ in the prefrontal cortex, amygdala, hypothalamus, and pituitary gland

As we intended to provide a comprehensive expression pattern of GRα and GRβ in the brain of P rats chronically exposed to ethanol, we measured the levels of total GR (*Nr3c1*), GRα and GRβ in the PL-PFC, $n = 7–8$ per group (Fig. 3A), IL-PFC, $n = 7–8$ per group (Fig. 3C), amygdala, $n = 8$ per group (Fig. 4B), and HPA sub regions such as the hypothalamus, $n = 7–8$ per group (Fig. 5A) and pituitary, $n = 8$ per group (Fig. 5D). Although chronic ethanol consumption did not significantly affect PL-PFC gene expression of total GR (*Nr3c1*), GRα, and GRβ, the changes in gene expression were in a similar trend as that observed in the NAc-core. Similarly, GRα/GRβ ratio was not significantly changed in PL-PFC between the ethanol-exposed group and the water-control group (Fig. 3A, $p > 0.05$). In the IL-PFC, the levels of total GR (*Nr3c1*) and GRα expression were not significantly changed by ethanol drinking (Fig. 3C, $p > 0.05$). Contrarily, GRβ levels were significantly reduced in the IL-PFC ($t(13) = 2.276$, $p \leq 0.05$) although the GRα/GRβ ratio remained unchanged. Total GR (*Nr3c1*), GRα, and GRβ gene expression as well as the GRα/GRβ ratio were not significantly altered in the amygdala, hypothalamus, and pituitary.

Effect of chronic ethanol exposure on GC-responsiveness in the NAc-core and NAc-shell

In order to examine the GC-responsiveness in each reward area of the brain, we used a commonly GC-induced gene, *Tsc22d3*, which is commonly referred to as glucocorticoid-induced leucine zipper (GILZ) (Hinds et al., 2011, 2014). GILZ is one of the GRα-targeting genes, which plays a role in host anti-inflammation and immunosuppression (Ng et al., 2017). Our results showed that GILZ expression was significantly downregulated in the NAc-shell of the ethanol-exposed group ($n = 7$) compared with the water-control group ($n = 6$), (Fig. 2F, $t(11) = 2.236$, $p \leq 0.05$). However, GILZ expression was not changed in the NAc-core ($n = 7–8$). These data suggest that the decrease in the GRα expression is associated with a reduction in its target gene GILZ; thus, decreasing the GC-responsiveness indicating GC-resistance.

Effect of chronic ethanol exposure on the CRH expression in the NAc-core, NAc-shell, PL-PFC, IL-PFC, amygdala, and hypothalamus

We next aimed to investigate the level of *Crh* mRNA expression following chronic ethanol drinking. We did not detect any significant changes in *Crh* levels of the NAc-core and NAc-shell (Fig. 2C, F), PL-PFC and IL-PFC (Fig. 3B, D), amygdala (Fig. 4C), and hypothalamus (Fig. 5B). The *Crh* was reduced in the IL-PFC and hypothalamus, although these reductions did not reach significance ($t(13) = 1.727$, $p > 0.05$) and ($t(13) = 1.847$, $p > 0.05$), respectively. It may be that longer chronic ethanol exposure may increase GC-sensitivity by either increasing GRα or decreasing GRβ, as GRβ was significantly lower in the IL-PFC and *Crh* was reduced.

Effect of chronic ethanol exposure on POMC mRNA expression in the hypothalamus and pituitary tissues

To address the role of POMC in ethanol dependence, we measured the *Pomc* mRNA expression in the hypothalamus and pituitary gland. Chronic ethanol consumption did not alter *Pomc* mRNA expression in these brain regions (Fig. 5C, E).

DISCUSSION

This study provides the first evidence that chronic ethanol exposure induced alterations in the expression of the GR isoforms in stress/reward brain neurocircuits. Despite the crucial role of the HPA axis and GR dysregulation during ethanol intoxication/withdrawal (Vendruscolo et al., 2012), the individual expression of GR isoforms in relation to ethanol intake and dependence has not been previously studied. We showed here that chronic ethanol consumption induced downregulation of GRα expression in the NAc-shell and hippocampus, and downregulation of GRβ in the NAc-core, hippocampus, and IL-PFC in alcohol-dependent P rats.

Previous studies reported that the GC resistance and reduced GRα/GRβ ratio had been associated with inflammatory diseases and mood disorders, such as schizophrenia, bipolar, and major depressive disorders [for review see (Lewis-Tuffin and Cidlowski, 2006)]. Interestingly, miR124 has been shown to be inversely associated with anxiety (Pan-Vazquez et al., 2015), and that miR124 targeting of GRα is involved in depression like behaviors

(Wang et al., 2017; Yi et al., 2018). Furthermore, lower GRα mRNA in the PFC, amygdala, and cingulate gyrus of postmortem brains have been revealed in major depressive disorder and suicide victims (Alt et al., 2010; Pandey et al., 2013). Our study reported differential effects of chronic ethanol exposure on the GR isoforms expression in several brain regions. We found a reduction in the expression of GRα and GRβ in the hippocampus. Since stress-related diseases are associated with dysregulation of GR activity (Ridder et al., 2005), our results suggest that the reduction of GR isoforms expression in the hippocampus may contribute to the neuropathology that might be caused by chronic ethanol consumption. Interestingly, we found that chronic ethanol consumption altered GR isoforms in a differential manner between the NAc-core and NAc-shell, i.e., reduction of GRα expression in the NAc-shell and reduction of GRβ in the NAc-core. The GRα expression being lower in the NAc-shell is likely due to higher miR124–3p levels. It is important to note that the NAc and PFC represent principal brain regions that control the reward-related behaviors. Glutamatergic projections from the IL-PFC to the NAc-shell are involved in the inhibition of the motivated reward-seeking behavior, and activation of this circuit results in the attenuation of reinstatement to cocaine-seeking elicited by associated cues (Keistler et al., 2015; Augur et al., 2016). Our results showed a decrease in the expression of GRβ in the NAc-core and GRα in the NAc-shell as well as higher GRα/GRβ ratio and thus GC sensitivity in the NAc-core but not in the NAc-shell. Studies demonstrated that antagonizing GC action with mifepristone attenuated ethanol drinking behavior (Vendruscolo et al., 2015). This suggests the potential role of GCs and stress in regulating ethanol-drinking behavior. The possibility of the increase in the GC activity in the NAc-core but not in the NAc-shell may contribute to the reinforcement of motivated seeking behavior by promoting the function of PL-PFC to NAc-core projections.

Lower GILZ expression further supports the decrease in the functional role of the GC system in the NAc-shell. As a GR target gene, GILZ mediates many GC functions such as the immune function, cytokine production, cell proliferation, and apoptosis (Delfino et al., 2004). It is known that suppression of GILZ expression is associated with the hyperinflammatory state; hence, GILZ expression is reduced in several inflammatory disorders, including Crohn’s disease, chronic rhinosinusitis, and atherosclerosis (Hahn et al., 2014; Ng et al., 2017). Lower GILZ expression was found to be linked to lower GRα expression, and higher miR124–3p. Albeit, GRα is lower in the PFC and central amygdala of depressive postmortem patients (Pandey et al., 2013), as miR124 has been found to be higher in depressive like behaviors (Wang et al., 2017; Yi et al., 2018). However, the GR isoform specificity was not measured. We found that GILZ expression was decreased in the NAc-shell but not in the NAc-core. This reduction was in accordance with GRα expression in NAc-shell i.e., decreases in the functional GC response. These alterations, however, were not associated with changes in the gene expression of CRH and POMC in these brain regions, which are suppressed by GCs as part of the HPA negative feedback (Kadmiel and Cidlowski, 2013).

The effect of chronic ethanol consumption on the CRH expression is well documented. CRH is believed to have a critical role in the development of drug dependence. A decrease in the hypothalamic CRH level was reported after seven days of exposure to high levels of ethanol vapors (Rivier et al., 1984), and in the hypothalamus of rats exposed to an ethanol-

containing liquid diet for two weeks (Redei et al., 1988). In accordance, lower CRH was found to be associated with higher ethanol craving (Olive et al., 2003). Alternatively, studies showed an increase in the rate of CRH biosynthesis and alteration in the ability of CRH to stimulate ACTH secretion after treatment with ethanol for several days (Rivier et al., 1990). Nevertheless, increases in the sensitivity to the locomotor-activating and anxiogenic-like effects of ethanol withdrawal may be mediated by extrahypothalamic CRH systems (Ehlers and Chaplin, 1987; Baldwin et al., 1991). Our study showed that chronic ethanol consumption didn't affect the levels of CRH in the hypothalamus and extrahypothalamic regions such as PFC, NAc, and amygdala. Our results are consistent with findings from a previous study that showed no changes in CRH mRNA levels in the hypothalamus after 14 days of ethanol administration (Zhou et al., 2000).

Regarding POMC, we showed no change in the expression of POMC mRNA in the hypothalamus and pituitary. It is important to note that studies have reported that 14 days ethanol exposure decreased the level of POMC mRNA in the pituitary gland (Dave et al., 1986), and 15 days ethanol exposure resulted in the increase in the POMC mRNA level in the hypothalamus (Angelogianni and Gianoulakis, 1993). However, ethanol withdrawal increased the level of POMC mRNA in the pituitary gland (Gianoulakis et al., 1988). Our results confirmed the previous finding that used 14 days of ethanol exposure (Zhou et al., 2000). However, Zhou and colleagues revealed a decrease in the POMC mRNA levels in the hypothalamus after one day of ethanol treatment, and this could be explained by the development of tolerance to ethanol consumption in the POMC-producing neurons. This may be explained by one-day ethanol induces GRα or lowers GRβ effecting the GRα/GRβ ratio, increasing sensitivity to GCs reducing POMC as negative feedback to the HPA axis, which maybe controlled by miR124–3p.

Serum corticosterone level was not changed in the chronic ethanol-exposed group as compared to the control group. Dynamic corticosterone level during ethanol consumption has a crucial effect on the development of neuroadaptation associated with ethanol dependence [for review, see ref. (Heilig and Koob, 2007)]. Plasma GC levels are increased during ethanol consumption and acute ethanol withdrawal (Adinoff et al., 1990); however, cortisol levels remain normal in chronic alcoholics in contrast to subjects who abstained from ethanol (Merry and Marks, 1972). In this regard, chronic ethanol consumption may normalize the levels of serum corticosterone and brain CRH and hence alleviate withdrawal-associated anxiety. It is possible that neuroadaptation occurred in chronic ethanol exposure paradigm, which led the animals to continue consuming ethanol. However, this is not excluding the possibility that chronic ethanol consumption may alter GC levels in stress/reward associated brain regions since previous studies showed that chronic ethanol consumption and withdrawal-induced variation between plasma and brain GC levels (Little et al., 2008).

Overall, our finding demonstrated that chronic ethanol consumption altered the expression of GRα and GRβ in brain regions involved in drug-seeking. The level of GILZ was changed in parallel with GRα function, which was most likely due to higher miR124–3p levels. Chronic ethanol consumption was found to be associated with normalized levels of serum corticosterone and brain CRH and POMC levels, which indicate the neuroadaptive effect

associated with chronic ethanol exposure. This study highlighted the role of GRα and GRβ and their possible advantage as therapeutic targets in the treatment of ethanol dependence.

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Abbreviations:

AUDs	Alcohol Use Disorders
BNST	bed nucleus of the stria terminalis
CRH	corticotrophin releasing hormone
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
HPA	hypothalamic–pituitary–adrenal
NAcc	nucleus accumbens core
NAcsh	nucleus accumbens shell
PFC	prefrontal cortex

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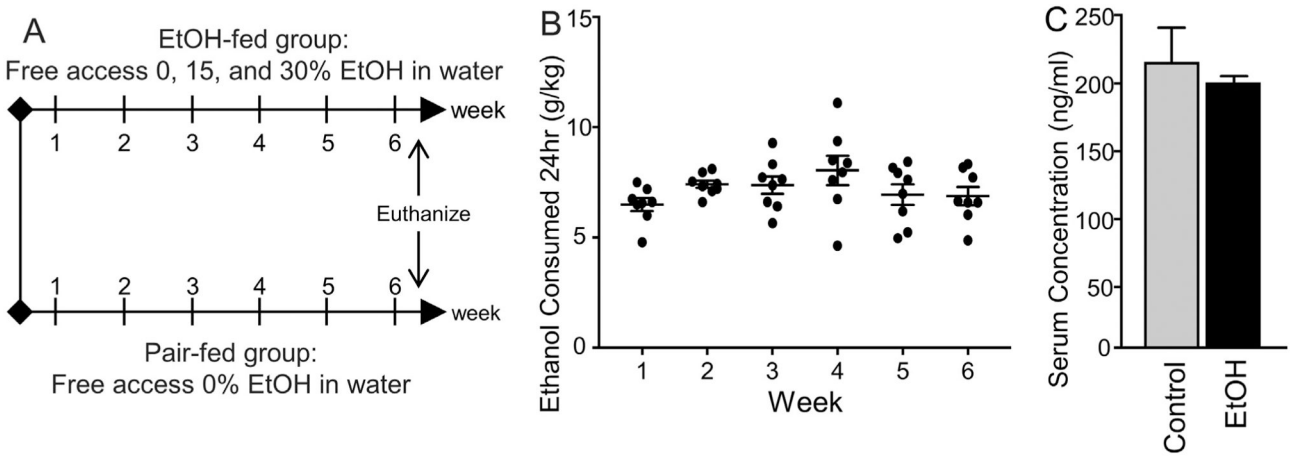

Fig. 1.

Ethanol consumption in P-rats and serum corticosterone level. **(A)** Chronic ethanol drinking paradigm: Adult male P rats were given free choice, continuous access to water, 15% and 30% ethanol for 6 weeks. The control group was given water only. Both control and ethanol groups were single housed with *ad libitum* access to food, and animals were euthanized at the end of week 6. **(B)** Ethanol intake (g/kg) over 6 weeks in P rats ($n = 8$) with continuous access to 15 and 30% ethanol in water. **(C)** Analysis of serum corticosterone levels in P rats. Unpaired *t*-test revealed no significant difference between the ethanol and water-control group (Mean ± SEM, $p > 0.05$).

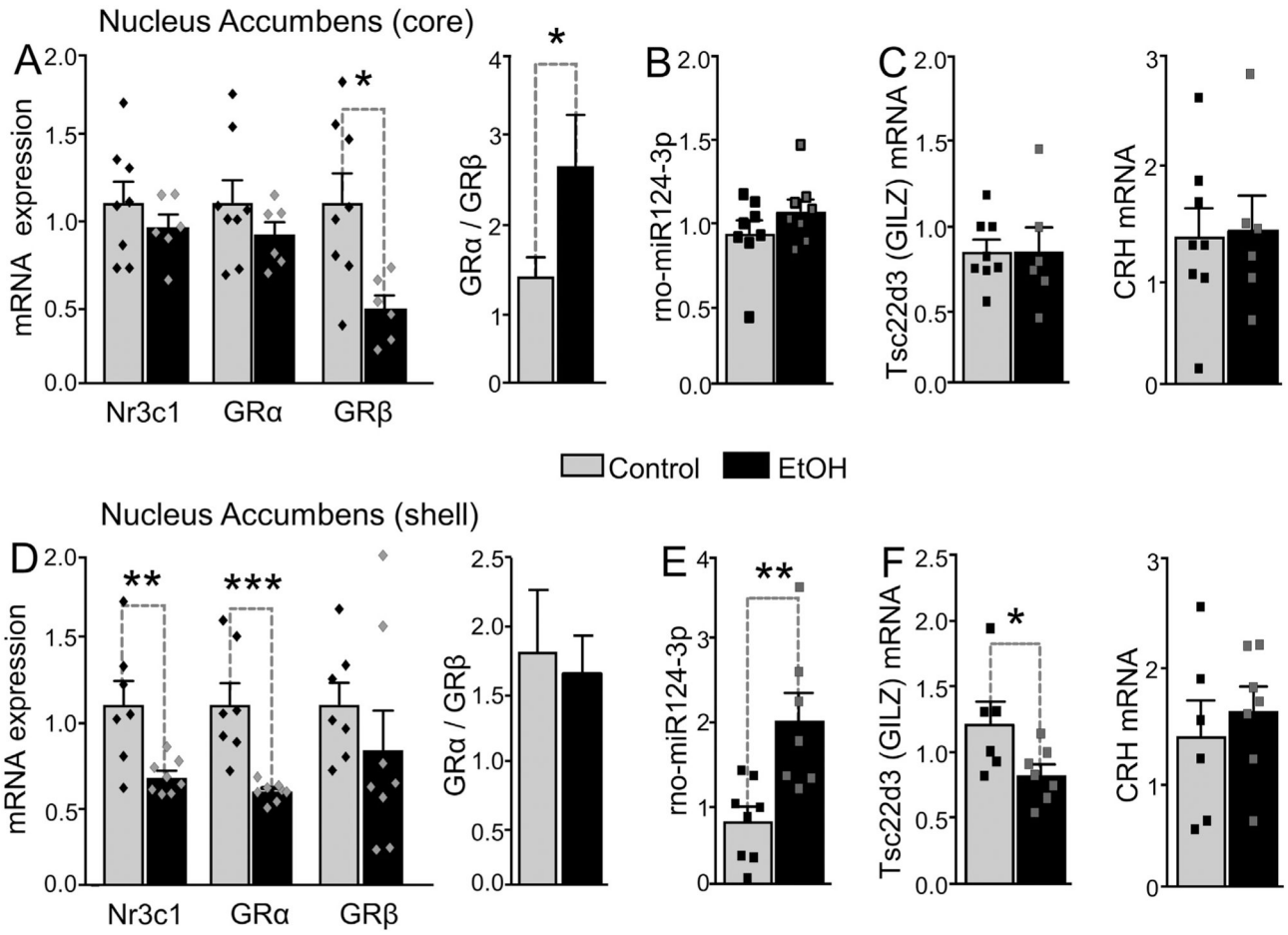

Fig. 2.

Expression of total GR, GRα, GRβ, CRH, and GILZ mRNA in the NAc-core and NAc-shell of the ethanol and water-control P rat groups. **(A)** Left: GRβ mRNA expression was significantly downregulated in the NAc-core of the ethanol group (unpaired *t*-test, Mean ± SEM, $p < 0.05$), while total GR was not changed. Right: GRα/GRβ ratio was significantly higher in the ethanol group compared with the water-control group (unpaired *t*-test, ± SEM, $p < 0.05$). **(B)** microRNA-124–3p (rno-miR124–3p) expression was not changed in the NAc-core. **(C)** *Tsc22d3* (GILZ) and *Crh* mRNA expression was not changed in the NAc-core. **(D)** Left: GRα mRNA level was significantly downregulated in the NAc-shell of the ethanol group compared with the water-control group (unpaired *t*-test, Mean ± SEM, $p < 0.001$), and total GR (*Nr3c1*) mRNA expression was significantly decreased (unpaired *t*-test, Mean ± SEM, $p < 0.001$) in the ethanol group as well. Right: the GRα/GRβ ratio was not changed in the ethanol vs the water-control group. **(E)** microRNA-124–3p (rno-miR124–3p) expression was significantly (unpaired *t*-test, Mean ± SEM, $p < 0.01$) increased in the NAc-shell. **(F)** *Tsc22d3* (GILZ) and *Crh* mRNA expression was not changed in the NAc-shell. (unpaired *t*-test, Mean ± SEM, $p < 0.05$).

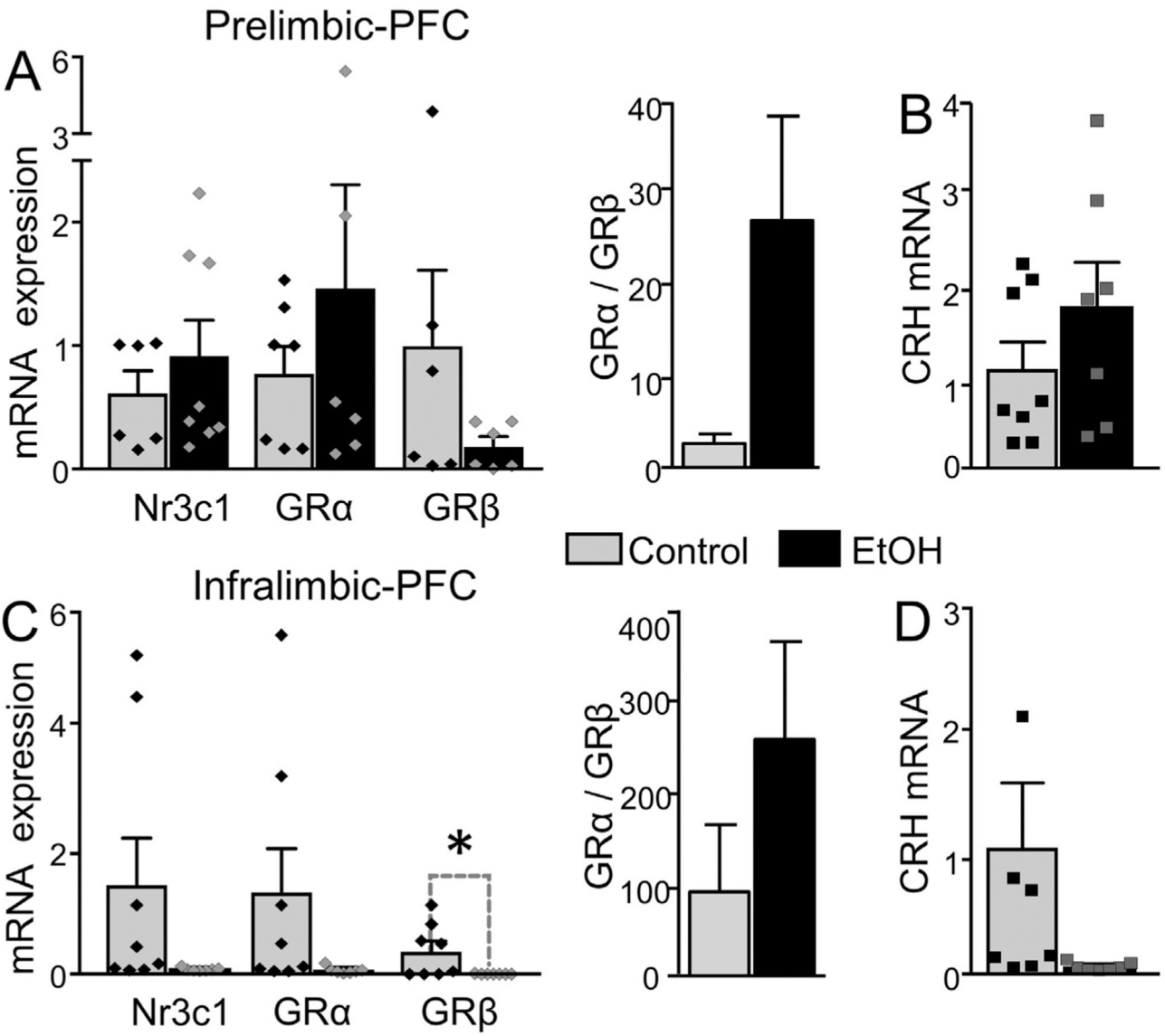

Fig. 3.

Expression of total GR, GRα, GRβ, and CRH mRNA in the PL-PFC and IL-PFC in ethanol and water-control P rat groups. **(A)** Left: GRα and GRβ mRNA expression were not significantly changed in PL-PFC of the ethanol group compared with the water-control group (unpaired *t*-test, Mean ± SEM, $p > 0.05$). Right: GRα/GRβ ratio was not significantly changed in the PL-PFC. **(B)** *Crh* mRNA expression was also not significantly changed in the PL-PFC of the ethanol vs control group. **(C)** Left: GRα mRNA levels did not change, whereas GRβ mRNA expression was reduced in the IL-PFC of the ethanol compared with the water-control group (unpaired *t*-test, Mean ± SEM, $p > 0.05$, $*p < 0.05$). Right: GRα/GRβ ratio was not significantly changed. **(D)** *Crh* mRNA expression was not significantly changed in IL-PFC of ethanol and control groups.

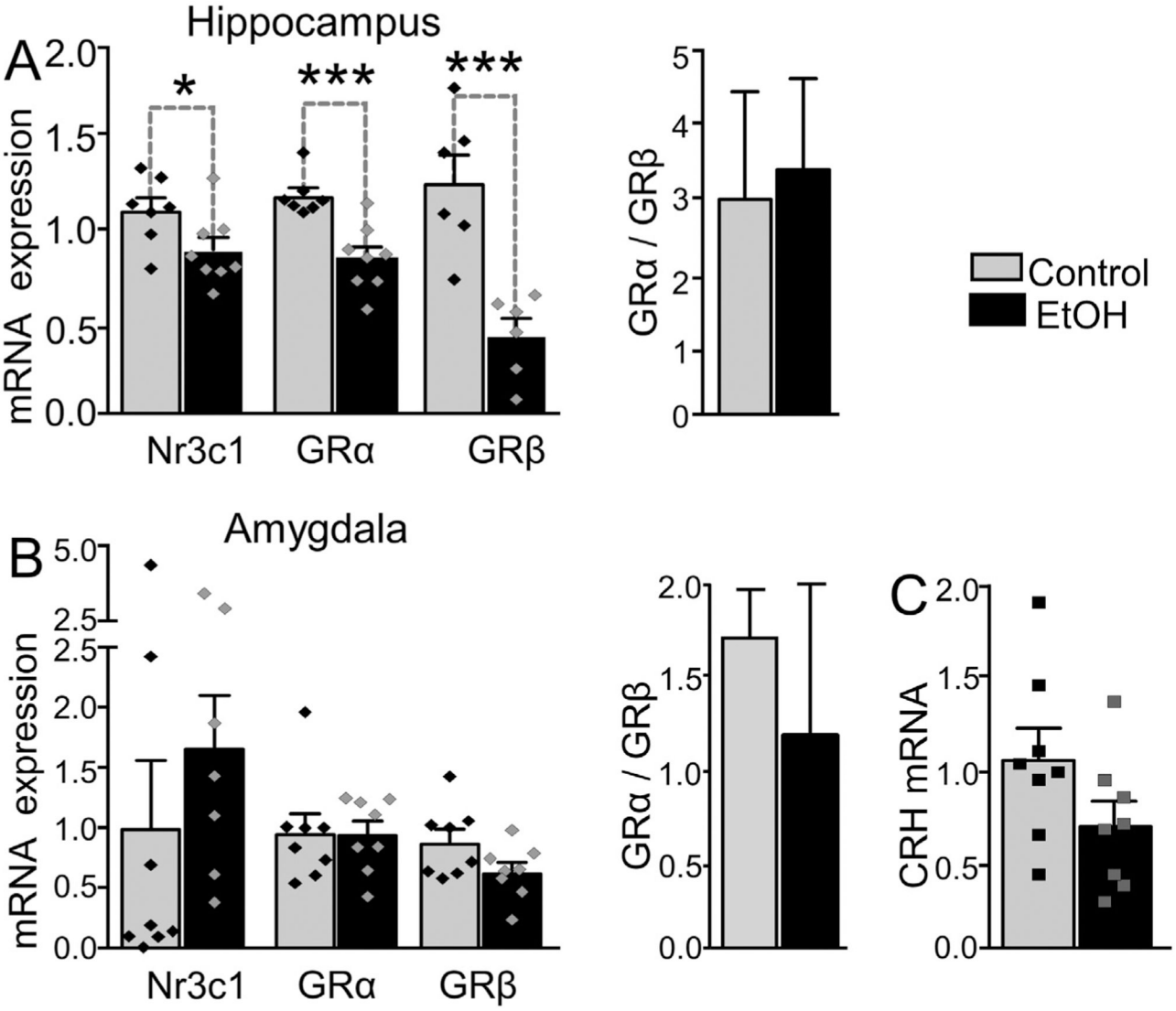

Fig. 4.

Expression of total GR, GRα, GRβ in the hippocampus and amygdala, in addition to CRH in the amygdala of ethanol-drinking and water-control rats. **(A)** Left: GRα, GRβ, and total (*Nr3c1*) GR mRNA expression were significantly downregulated in the hippocampus of the ethanol group as compared with the control group (unpaired *t*-test, Mean ± SEM, $p < 0.05$). Right: The GRα/GRβ ratio was not changed in the ethanol group vs the water-control group. **(B)** GRα, GRβ, and total (*Nr3c1*) GR mRNA expression were unchanged in the amygdala of the ethanol group compared with the water-control group (unpaired *t*-test, Mean ± SEM, $p > 0.05$). **(C)** *Crh* mRNA expression was not changed in the amygdala.

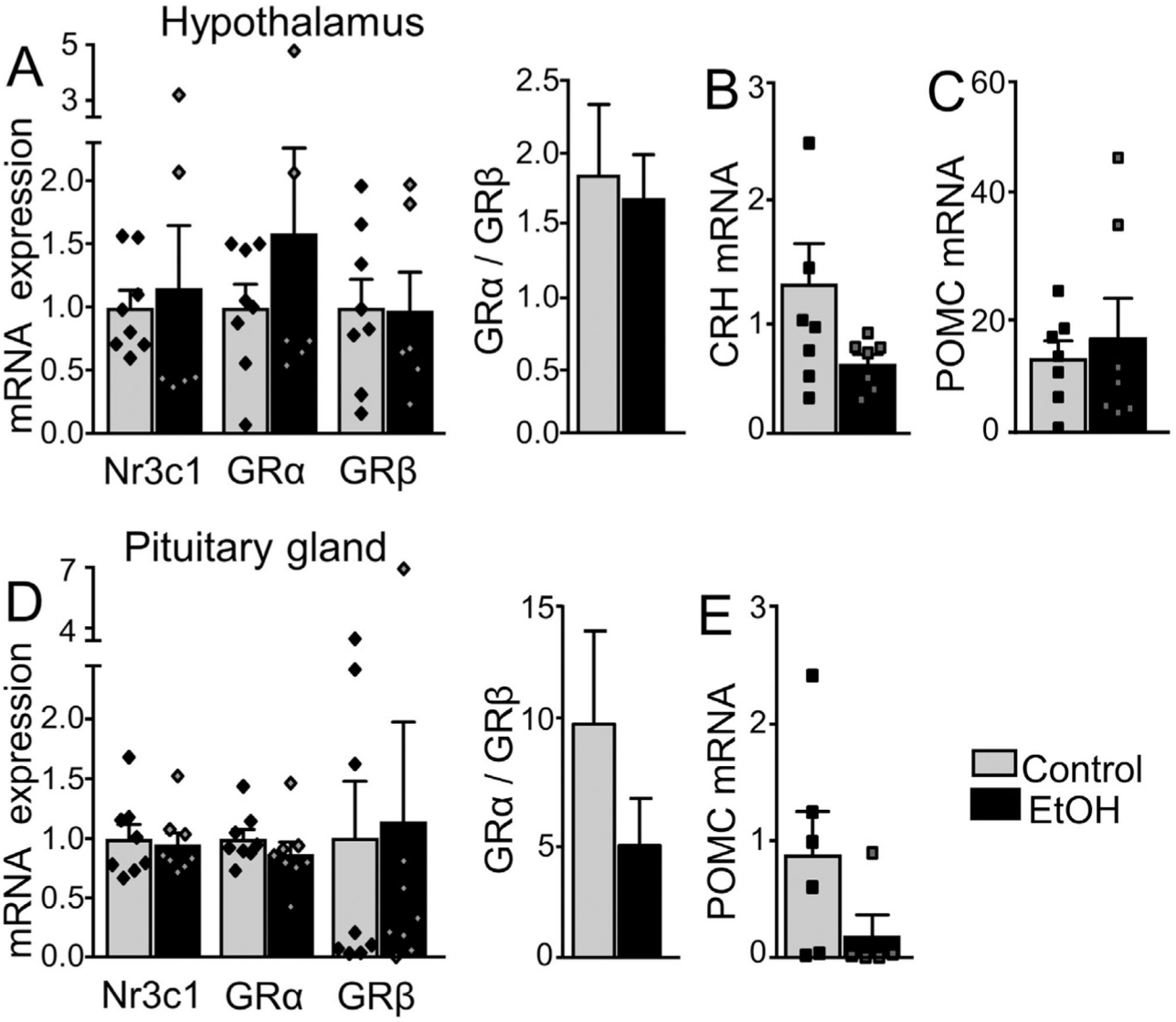

Fig. 5.

Expression of total GR, GRα, GRβ, and POMC in the hypothalamus and pituitary gland, as well as CRH in the hypothalamus of the ethanol-drinking and water-control rats. **(A)** Left: total (*Nr3c1*) GR, Grα, and GRβ mRNA expression were not significantly changed in the hypothalamus (unpaired *t*-test, Mean ± SEM, $p > 0.05$). Right: GRα/GRβ ratio was not changed in the ethanol group compared with the water-control group. **(B)** *Crh* mRNA expression was not significantly changed in the hypothalamus following ethanol consumption. **(C)** *Pomc* mRNA expression in the hypothalamus was not significantly changed by ethanol drinking. **(D)** Left: GRα and GRβ mRNA expression were not significantly changed in the pituitary gland of the ethanol-drinking group vs the water-control group (unpaired *t*-test, ± SEM, $p > 0.05$). Right: GRα/GRβ ratio remained unchanged in the ethanol group compared with the water-control group. **(E)** *Pomc* mRNA

expression was not significantly changed in the pituitary gland following chronic ethanol drinking.