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Chronic Ethanol Exposure Increases the Association of Hippocampal Mu-Opioid Receptors with G-Protein Receptor Kinase 2 (GRK2)

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Abstract

Opioid receptors (ORs) have been shown to have a significant role in the central nervous system (CNS) effects of chronic ethanol consumption. The OR antagonist, naltrexone, is used clinically to reduce continued intake. We previously observed that chronic ethanol consumption, by adult male Sprague-Dawley rats, induced a reduction in functional coupling of mu and delta ORs to G-proteins in rat CNS regions, including the hippocampus (Saland et al., 2004). G-protein receptor kinase (GRK) 2 phosphorylates G-protein coupled receptors, including ORs, after agonist binding, as part of normal regulation and desensitization. We tested the hypothesis that chronic ethanol exposure affects the association of the GRK2 with the mu OR. Co-immunoprecipitation methods were used to determine if mu OR association with GRK2 is elevated in the hippocampus after chronic ethanol, when compared to controls. Hippocampal homogenates from chronic ethanol and pair-fed control rats were treated with affinity-purified rabbit polyclonal antibodies (ab) to mu OR, and immune complexes were probed for GRK2 by immunoblotting techniques. Results demonstrate an association of GRK2 with mu ORs in chronic ethanol-treated rats, but not in the controls. Possible changes in GRK2 association with ORs after chronic ethanol may be related to levels of phosphorylation and subsequent trafficking of the receptors.

Keywords

opioid receptors; chronic ethanol; G-protein receptor kinase; immunoprecipitation

Introduction

Chronic consumption of ethanol affects ORs in the CNS in laboratory animals and humans (Becker et al., 2002; Froehlich et al., 1998; Herz, 1997; Roberts et al., 2001; Roberts et al., 2000; Soini et al., 2002). While ethanol does not directly bind to ORs, it is known to acutely stimulate release of CNS endogenous opioids, which then can bind to ORs (Froehlich, 1997; Gianoulakis, 2004; Herz, 1997). After chronic ethanol, the latter authors note that there is a reduction in endogenous opioid release, and the ORs appear to adapt, in expression, as well as in the ability to couple to G-proteins, as described in prior studies noted below.

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Opioid agonist stimulation of the ORs results in coupling to Gi/o proteins, followed by phosphorylation and internalization, which are events directly related to uncoupling from the G proteins (Chen and Lawrence, 2000; Law and Loh, 1999). Phosphorylation of ORs is mediated by a number of protein kinases, including G-protein receptor kinases, such as GRK2 (Wang and Wang, 2006). In addition, phosphorylation of ORs increases the affinity of the protein β -arrestin for the receptor, thereby leading to further uncoupling, followed by internalization and sequestering of the receptors within the cell. Both GRKs and β -arrestin are thought to be part of the mechanisms for agonist-dependent sensitization and internalization of mu ORs (Hurle, 2001).

Several studies have shown that chronic ethanol consumption in rat animal models reduces functional coupling of mu ORs to G-proteins in brains of alcohol-preferring rat strains (Chen and Lawrence, 2000; Sim-Selley et al., 2002), as well as both mu and delta receptors of multiple brain areas, including hippocampus, in ethanol non-preferring Sprague-Dawley animals (Saland et al., 2004). However, the mechanisms which underlie the reduced coupling (uncoupling) of the ORs, after ingestion of chronic ethanol, have not been examined. It is possible that reduced OR coupling under the latter conditions could be related to changes in phosphorylation of ORs.

While there are many studies on phosphorylation of ORs, and the relevant kinases, including GRKs, after binding of receptor- selective agonists, to our knowledge, there is very little information available on the GRKs which may phosphorylate ORs in the CNS after ethanol intake. Narita and colleagues (Narita et al., 2007) studied neuropathic pain in rats withdrawn after chronic ethanol, and observed reduced levels of morphine-induced pain suppression, as well as decreased coupling of mu ORs to G-proteins, in spinal cord membranes. The authors observed increased levels of phosphorylated protein kinase C (PKC) in spinal cord after morphine treatment, but no change from controls in levels of GRK2 or protein kinase A (PKA), using Western blot methods.

Our present studies address the potential direct association of mu ORs with the G-protein coupled receptor (GPCR) phosphorylating enzyme GRK2, by the use of co-immunoprecipitation methods, in the hippocampus of Sprague-Dawley rats, after chronic ethanol consumption. The hippocampus was selected based upon our prior observations of reduced mu and delta OR agonist- stimulated G-protein coupling in both CA1 and the dentate gyrus (Saland et al., 2004). We also previously observed reduced immunohistochemical expression of mu ORs after chronic ethanol, in multiple brain areas, including hippocampus, with the same animal protocol (Saland et al., 2005).

An association of GRK2 with the mu OR was demonstrated in the tissue from the ethanol animals. To our knowledge, this is the first evidence of a co-association of the mu OR with GRK2 in a region of the brain, after chronic ethanol ingestion.

Materials and Methods

Animal experimental protocols

All animal experiments were authorized by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee, and performed in accordance with National Institutes of Health guidelines. Adult male Sprague-Dawley rats, weighing approximately 250 g, were obtained from Harlan Industries (Indianapolis, IN), and allowed to acclimate to the University of New Mexico School of Medicine Animal Resource Facilities environment for one week prior to initiation of the diets described below. Rats were individually housed in standard cages and bedding in a room maintained at 22 °C, on an 8-hour dim light/16-hour dark cycle (lights on at 08:30). This on-off timing protocol, with a

longer dark cycle, was used to allow more access to the liquid diet. This timing was used initially (Ferreira et al., 2001) to study glutamate receptor expression after long-term ethanol exposure, and was also used previously by our laboratory (Saland et al., 2004; Saland et al., 2005).

After acclimation, rats were divided into the ethanol-ingesting and pair-fed groups, as described in detail previously (Ferreira et al., 2001). Rats received a Bioserv (Frenchtown, NJ) chocolate-flavored liquid diet based on the Lieber and DeCarli formulation (Lieber and DeCarli, 1982). This liquid diet provides 1 Kcal/mL. One group of rats (ethanol-treated group) were offered 80 mL of diet containing 0% v/v (days 1–2), 3% v/v (days 3–4), 5% v/v (days 5–7) and 6.7% v/v ethanol (days 8–16). Rats treated with the same diet have previously been shown to consume ~80 mL/day during days 1–4, ~60–70 mL/day during days 5–7, and ~40–65 mL/day during days 8–16 (Ferreira, 2001). A second group of rats (pair-fed controls) were given equivalent amounts of the liquid diet without ethanol, which was made isocaloric to the ethanol-containing diet by the addition of maltose-dextrins. The liquid diet was available to the rats during the dark cycle, and water bottles were removed from the cages during this period. The liquid diet tubes were removed each morning and the water bottles returned to the cages. Tubes were removed in the morning because rats are nocturnal animals that do not consume significant amounts of this liquid diet during the light cycle (Savage et al., unpublished observation). Rats had free access to water during the light hours to prevent dehydration. Liquid diet consumption was checked daily. There was no significant difference in body weight between control and ethanol-fed animals.

Tissue preparation and immunoprecipitation protocols

On day 16, rats were sacrificed by rapid decapitation without anesthesia at the peak of alcohol consumption. Trunk blood was collected and alcohol levels were determined by the alcohol dehydrogenase method, as previously described (Saland et al., 2004; Saland et al., 2005). Brains were removed, and the hippocampus dissected and frozen in liquid nitrogen, then stored at -80°C until used.

Tissue preparation—Hippocampal tissue (whole hippocampal formation) from chronic ethanol or pair-fed control rats ($N = 4$ pairs of animals) and frozen at -80°C , as described above, was thawed on ice. Samples were homogenized by sonication for one to two minutes in phosphate-buffered saline plus a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined by the Lowry assay (Lowry et al., 1951). Homogenates were stored at -80°C until used. Hippocampal homogenates (200 μL per sample, 0.2 mg/mL protein concentration) from chronic ethanol or pair-fed control animals, were thawed, and Triton X-100 (final concentration of 1% v/v) and 10 μL undiluted affinity-purified rabbit anti-mu opioid receptor antibody (Biosource, Camarillo, CA) were added. Samples were incubated with mixing overnight at 4°C . Additional tissue samples were incubated with 5 μL of rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, 400 $\mu\text{g/mL}$), **or** without inclusion of antibody or IgG. After overnight incubation, 30 μL packed volume of protein A-sepharose beads in homogenation buffer plus Triton-X-100 (1% v/v) was added, and the incubation continued for 90 min. at 4°C . The sepharose beads were collected by centrifugation (30 sec., 12,000 $\times g$, 4°C) and washed twice with 900 μL of buffer (20mM Tris-HCl, pH7.4, 1 mM EDTA, 320 mM sucrose, 75 mM NaCl, 75 mM KCl, 16 mM (1% v/v) Triton X-100, 20 mM β -glycerophosphate, 20 mM sodium pyrophosphate, and 10 mM sodium fluoride). After discarding the final wash, 30 μL of SDS-PAGE sample buffer was added, followed by brief vortexing, then boiling for two minutes. Following a 30 second spin at 12,000 $\times g$, the eluted material was used for Western blot analysis, as described below.

Western blot protocol

Samples (10–15 μ L loaded) of anti-mu OR immune complexes, as well as rabbit IgG and no antibody control samples, isolated from hippocampus of paired animals, that is, from one chronic ethanol-treated rat and its pair-fed control, were separated on pre-cast polyacrylamide gels of concentrations ranging from 7.5 to 12 % (Tris-HCl Ready Gels, BioRad, Hercules, CA), and electrotransferred to nitrocellulose membranes. Non-specific binding to the membranes was prevented by blocking with fresh 10% (w/v) nonfat dry milk plus 0.4% (w/v) Tween-20, for 2 hours at room temperature, or overnight at 4 °C. Membranes were probed with primary mouse antibody to GRK2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. The membranes were washed, then probed with horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody (1:1000, Chemicon, Temecula, CA). Detection of the bound antibodies was performed by chemiluminescence using a kit from Roche Biochemicals (Indianapolis, IN). For analyses of total GRK2 in homogenates of hippocampus of chronic ethanol and pair-fed control animals, Western blots were performed using additional samples from four (4) pairs of animals.

For detection, Fuji medical X-ray film (Tokyo, Japan, #100NIF) was exposed to the membrane in an X-ray cassette, then developed using a Konica SRX-101A film processor. Images of blots were scanned in a Hewlett-Packard Scanjet II cx. Blots were quantified using BioRad Quantity One 4.2.1 software.

For the immunoprecipitations, comparisons were made between the mean ratio of the optical densities (O.D.) of anti-GRK2 immunoreactive bands present in ethanol or control tissue samples incubated with anti-mu OR antibody(ab) and the bands of ethanol or controls treated with IgG (as an example: O.D. ethanol +ab/O.D. ethanol +IgG). For Western blots of total GRK2, comparisons were made between the mean optical densities of ethanol versus control anti-GRK2 immunoreactive bands which were normalized to the mean intensity of two protein bands, adjusted for background, after staining the membranes with Coomassie Brilliant Blue. Data was analyzed via GraphPad Prism software, using a two-tailed unpaired Student's t-test. The accepted level of significance was $p < 0.05$.

Results

Immunoprecipitation of hippocampal tissue from pair-fed control and ethanol-treated rats, with antibody to the mu-opioid receptor, followed by Western blot analysis, using antibody to GRK2, as described in the Methods, yielded a band at 82.5 kDa. Figure 1 A illustrates a representative blot shown from one pair of animals. The lower band at approximately 50 kDa represents the mu OR antibody used for immunoprecipitation. In samples in which the tissue was incubated with rabbit IgG, the 82.5 kDa band represents non-specific binding (trapping) of GRK2 to the sepharose beads and the secondary antibody binding. In samples in which the mu OR antibody was omitted, the lower antibody band is absent.

We observed that the mean ratio of the optical density (O.D.) of the ethanol+antibody (ab) GRK2 bands to the ethanol+IgG bands was significantly higher than the mean ratio of the O.D. of control+ab/control IgG ($p < 0.0002$). Samples incubated without mu antibody or rabbit IgG show a band that represents non-specific binding of GRK2 to the protein A-sepharose beads used in the immunoprecipitation reaction. Data are summarized in Figure 1 B.

To determine if there was a difference in total GRK2 expression in chronic ethanol versus control animals, we measured anti-GRK2 immunoreactivities present on Western blots of whole hippocampus, using tissue samples from four pairs of animals. The mean O.D. of the GRK2 bands for the ethanol animals was not significantly different from controls. Figure 2 A illustrates a representative blot from one animal pair, and data are summarized in Figure 2 B.

Discussion

The present study demonstrates an association of mu OR with GRK2 in the hippocampus of chronic ethanol-ingesting rats (Figure 1). In contrast, we were unable to detect a significant association between mu OR and GRK2 in control hippocampus, indicating either that it was below the limits of our detection in controls, or the association is specific to chronic ethanol-treated animals. Our prior studies with the same chronic ethanol animal model found that there was reduced coupling (“uncoupling”) of both mu and delta ORs to G-proteins, in several brain areas, including the hippocampus (Saland et al., 2004). The results of the present study indicate that a mechanism for the reduction in functional coupling of ORs could possibly be enhanced association of ORs with GRK2, and subsequent phosphorylation of the receptors after continued ethanol intake.

It is unlikely that the observed effect of chronic ethanol on co-association of GRK2 and the mu OR is a result of increased expression of the mu OR or GRK2. As noted above in the Introduction, we have previously observed reduced expression of immunoreactive mu ORs in the hippocampus and multiple other CNS areas, in the ethanol-ingesting animals, as compared to controls (Saland et al., 2005), using fluorescent histochemical methods on tissue sections which were stained with the same antibody for mu ORs as in the current study. In addition, in the current study, we observed that total GRK2 did not differ between ethanol and control animals in routine Western blots.

Further evidence for the importance of GRKs in regulation of ORs comes from studies on morphine effects in rat brain, where GRK2 and GRK5 messenger ribonucleic acids (mRNAs) were increased in several rat brain regions, including hippocampus, after acute morphine injections. The GRKs were down-regulated with chronic morphine, with aberrant increases after withdrawal (Fan et al., 2002). Other authors (Ferrer-Alcon et al., 2004) found that human postmortem prefrontal cortex taken from opiate addicts showed decreases in immunoreactive glycosylated mu ORs, as well as in GRK2 and 6 and beta-arrestin-2, using Western blots. However, McLaughlin and co-authors (McLaughlin et al., 2004) observed that agonist stimulation of the kappa OR, for analgesia to pain in mice, could produce prolonged phosphorylation of the receptor, with tolerance developing and being maintained as long as the receptor remained phosphorylated. The tolerance and prolonged phosphorylation of the receptor were suggested to be mediated by GRK.

Overall, the mechanisms described above suggest that under basal circumstances, opioid ligands are thought to bind to receptors, followed by G-protein coupling to Gi/Go proteins, and inhibition of adenylyl cyclase. Subsequently, to down-regulate and stop the continued stimulation of the receptors, there is phosphorylation mediated by GRKs, binding of the phosphorylated receptors by arrestins, and internalization and ultimate recycling or sequestration of the receptors, to begin the cycle again.

In conclusion, our present observations suggest that GRK2 will bind to the mu OR in the hippocampus of chronic ethanol-ingesting animals. An association of GRK2 with the receptor would offer a mechanism for receptor phosphorylation, which would, in turn, contribute to a reduction in opioid-receptor-induced G-protein coupling in the chronic ethanol animals as compared to controls, as has been shown in prior investigations cited above. Additional studies planned will extend beyond chronic ethanol ingestion, to examine the effects of withdrawal, followed by relapse, on functional coupling, phosphorylation, and internalization mechanisms of CNS opioid receptors.

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Control and Ethanol Hippocampus Immunoprecipitation with MuOR, probe for GRK2

Figure 1. Co- Immunoprecipitation of mu OR with GRK2 in chronic ethanol and pair-fed control rats

A. Immunoprecipitation of chronic ethanol (E9+ab) and its pair-fed control animal (C9+ab) rat hippocampal tissue with affinity-purified rabbit anti-mu opioid receptor (OR) antibody, followed by Western blot of the immunoprecipitate, with mouse anti-GRK2. E9+IgG and C9+IgG lanes contain samples from the same two animals immunoprecipitated with rabbit IgG. Additional lanes contain samples treated without antibody or IgG (E9- and C9-). Note GRK2 immunoreactivity (band at 82.5 kDa) in all lanes with tissue. The lower band at approximately 45 kDa represents the secondary antibody binding to the mu OR antibody used for immunoprecipitation, and a faint lower band in samples containing IgG.

B. Graph of Western blot data from immunoprecipitation of tissue from 4 pairs of animals. Data expressed as the optical density (O.D.) ratio of the GRK2 band of control or ethanol samples plus mu antibody to those plus IgG. The mean O.D. ratio of the E+ab/E+IgG is significantly higher ($p<0.0002$) than C+ab/C+IgG.

Figure 2. Western blot of GRK2 in chronic ethanol and pair-fed control rats

A. Representative Western blot of hippocampal tissue from one pair of animals (control animal #1 and ethanol animal #1) prepared as described in the Methods. A single band at 82.5 kDa represents GRK2.

B. Graph of Western blot data from 4 pairs of animals. There is no significant difference between the optical density (O.D.) of the bands between the ethanol and control animals.