The Effect of Chronic Ethanol Consumption and Withdrawal on μ-Opioid and Dopamine D₁ and D₂ Receptor Density in Fawn-Hooded Rat Brain

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ABSTRACT

Previous studies have implicated the dopamine and opioid systems in the induction and maintenance of ethanol consumption. This study investigated, in alcohol-preferring Fawn-Hooded (FH) rats, whether chronic free-choice ethanol consumption and subsequent withdrawal cause alterations in central μ-opioid, dopamine D₁, and D₂ receptor density using autoradiography. FH rats were given a free choice between a 5% ethanol solution and tap water (n = 25) and displayed a mean ethanol consumption of 5.6 g/kg/day. A parallel group of FH rats (n = 5) only had access to tap water. Rats were then withdrawn from ethanol for 0, 1, 2, 5, or 10 days and killed by cervical dislocation and decapitation. Increases in μ-opioid receptor density were observed in the nucleus accumbens and ventral tegmental area upon withdrawal compared with the ethanol naive group. In the lateral amygdala, binding in all withdrawal groups was significantly different from the ethanol naive FH rats, and also from the chronic ethanol rats. An increase in dopamine D₁ receptor density was observed in the substantia nigra, pars reticulata in the 5- and 10-day withdrawal groups compared with ethanol naive. Accumulant dopamine D₂ receptor density (+25–30%) increased in the 10-day withdrawal group compared with both naive and chronic ethanol groups. These findings demonstrate that the opioid and dopamine systems are susceptible to modulation by chronic ethanol consumption and withdrawal in the FH rat. Furthermore, although acute ethanol withdrawal results in modulation of μ-opioid receptors, effects on dopamine receptors are delayed and only become evident 5 to 10 days after withdrawal.

Alcohol is one of the most widely used recreational drugs in the world; however, “alcohol causes as much death and disability as measles and malaria and far more years of life lost to death and disability than tobacco or illegal drugs” (World Health Organization, a summary of global status report on alcohol; http://www.who.int/substance_abuse/who_ncd_msd_2001_2.pdf). Because alcohol abuse can cause significant social, economic, and personal damage, a vast amount of research has gone into understanding the ways in which ethanol interacts with the central nervous system to cause addictive behavior. Initially, drinking alcohol is associated with an elevation of mood and euphoria, which are components of positive reinforcement. However, once alcohol-seeking behavior has been established, periods of withdrawal are marked by feelings of craving and discomfort, which may serve as a cue to reinstate drug-taking behavior. Past research has strongly implicated the mesolimbic dopamine system in reward and drug-seeking behavior (Diana et al., 1998). A major component of this drug reward circuit is the dopaminergic connection between the ventral tegmental area (VTA) and the basal forebrain, which includes the nucleus accumbens (NAcc) and amygdala (Bardo, 1998). Many drugs of abuse either directly or indirectly facilitate dopamine release in the nucleus accumbens (Bardo, 1998).

Evidence also suggests that activation of the endogenous opioid system after consumption of ethanol may be part of a neurobiological mechanism that is involved in ethanol reinforcement and high ethanol consumption. Importantly, opioid peptides are found in key regions of the mesolimbic system, the main network thought to be involved in ethanol-seeking behavior (Mansour et al., 1994; Froehlich et al., 1996). Opioid neurotransmission in the reinforcing effects of ethanol seem to be supported because naltrexone, an opioid

ABBREVIATIONS: VTA, ventral tegmental area; NAcc, nucleus accumbens; FH, Fawn-Hooded; GTPγS, guanosine-5’-O-(3-thio)triphosphate; CPu, caudate putamen; BSA, bovine serum albumin; ANOVA, analysis of variance; SNr, substantia nigra, pars reticulata; EP, entopeduncular nucleus; Nsc, substantia nigra, pars compacta; FK 33,824, (D-Ala², N-Me-Phe⁵, methionin (O-ω)³)-enkephalin; NCQ 298, (S)-3-iodo-N-[1-ethyl-2-pyrrolidinyl][methyl]-5,6-dimethoxysalicylamide; SKF 77473, +7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SCH 23982, 8-iodo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol.
receptor antagonist, has been shown to reduce alcohol intake in humans (Volpicelli et al., 1992). Studies have also shown that dopamine, acting at D2 receptors, negatively regulates levels of enkephalin mRNA in the NAcc (Hong et al., 1980). This can be explained with respect to the output pathways from the NAcc to the mesencephalon. The indirect pathway involves striatopallidal neurons, which project from the NAcc to the VTA via the ventral pallidum and express enkephalin and dopamine D2 receptors. In contrast, the direct pathway involves striatogeminal neurons projecting directly from the NAcc to the VTA, expressing dynorphin and dopamine D1 receptors (Spanagel et al., 1992).

Previous studies have investigated the effect of abused substances on μ-opioid receptor density in various regions of the rat brain. More specifically, it has been shown that after withdrawal of administered morphine in Wistar Kyoto rats, there is a decrease in the density of μ-opioid receptors in the nucleus accumbens and striatum after 24, 48, and 96 h, however, no changes after ethanol withdrawal (Türchan et al., 1999). In Wistar rats, systemic administration of ethanol results in acute alterations of μ-opioid receptor density, with an initial decrease in binding in the VTA and NAcc, along with an increased binding in frontal and prefrontal cortex (Méndez et al., 2001). Importantly, these studies are examining the effect of administered ethanol in rats that do not possess an alcohol-prefering phenotype, which is not a true examination of reward-related neural adaptation. In other rats such as the Fawn-Hooded (FH) strain, voluntary ethanol consumption alone has been shown to cause bidirectional changes in μ-opioid receptor density dependent on brain region (Cowen et al., 1999). Furthermore, acute withdrawal after chronic free-choice ethanol consumption in FH rats results in increased levels of μ-opioid receptor agonist-stimulated [35S]GTPγS binding in the amygdaloid complex, NAcc, and striatum (CPu) compared with chronic ethanol (Chen and Lawrence, 2000), suggestive of receptor adaptation during the early phases of withdrawal. One could therefore hypothesize that acute (1–2 day) withdrawal may result in up-regulation of μ-opioid receptors in these reward-related structures. The effect of longer periods of withdrawal after volitional consumption of ethanol on these receptors has not yet been investigated in alcohol-prefering rat strains such as the FH rat. This particular strain of rat consumes large quantities of ethanol in a free-choice situation (Rezvani et al., 1990; Chen et al., 1998) and is thus useful in exploring the effects of reward rather than the effects of noncontingent administration of ethanol.

Given the evidence of the interaction between the dopamine and opioid systems in the induction and maintenance of ethanol consumption (Cowen and Lawrence, 1999), the aim of this study was to extend past research and investigate whether enforced withdrawal after chronic consumption of ethanol leads to alterations in dopamine D1, D2, and μ-opioid receptor density after chronic ethanol consumption and investigate whether this is region-specific.

**Experimental Procedures**

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

**Materials.** FK 33,824 ([t-Ala²,N-Me-Phe⁴, methionin (O-ol)-]enkephalin) was obtained from Bachem (Bubendorf, Switzerland). Raclopride and NCQ 634 (des-iodo derivative of NCQ 298) were gifts from Astra (Häsele, Möndal, Sweden). Naloxone HCl was purchased from Endo Laboratories (NY) and 125I-T9SC 23982 (2200 Ci/mmol) purchased from PerkinElmer Life Sciences (Boston, MA), whereas SKF 77434 was purchased from Sigma/RBI (Natick, MA). 125I-Na (200 Ci/mmol) and LM1 emulsion were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Bovine serum albumin (BSA) (peptidease free) was from Sigma-Aldrich (St. Louis, MO) and BSA (analytical grade) from the Commonwealth Serum Laboratories (Parkville, VIC, Australia). Films (X-omat AR) used for autoradiography were purchased from Eastman Kodak (Rochester, NY). All other chemicals and reagents were purchased from various sources and were either laboratory or analytical grade.

**Ethanol Consumption.** Male FH rats were bred from stock parents obtained from Dr. Amir Rezvani (University of the North Carolina School of Medicine, Chapel Hill, NC). Rats (7–8 weeks old) were randomly assigned to six groups: ethanol naive; chronic ethanol; or 1-, 2-, 5-, or 10-day withdrawal. For daily fluid consumption monitoring, rats were individually housed in a 12-h light/dark cycle with free access to standard chow. Each cage was equipped with two drink bottles, either both filled with tap water or one filled with tap water and the other with 5% ethanol (n = 25). After acquisition of stable ethanol consumption (~1 week), respectively, the respective drink containers were weighed each day for a further 5 weeks to determine daily consumption rates of both water and 5% ethanol. All data presented represent consumption rates during this 5-week period of two-bottle free-choice ethanol. Drink container positions were changed randomly to prevent the development of place preference. Ethanol was subsequently withdrawn from the rats, and at 0, 1, 2, 5, or 10 days after withdrawal, rats were killed by cervical dislocation and decapitation, the brains quickly removed and frozen over liquid nitrogen, and stored at ~80°C until use.

**Autoradiography.** Horizontal sections of cytocast-cut (Cryocut 1800; Leica, Wetzlar, Germany) rat brain (14 mm) were thaw mounted onto gelatin-chrome alum-coated slides and stored at ~80°C until use. On the day of the experiments, tissue sections were removed from ~80°C storage and allowed to warm to room temperature.

**μ-Opioid Receptors.** The Chizzonite method (Cowen et al., 1999) was performed to iodinate FK 33,824, a selective μ-opioid receptor agonist. To determine μ-opioid receptor binding, slides were incubated in 50 mM Tris-HCl, pH 7.4, containing 0.1% BSA and 0.1 nM 125I-FK 33,824 (2000 Ci/mmol) for 60 min at room temperature. Nonspecific binding was determined in the presence of 10 μM naloxone. At the end of the incubation period, slides were subjected to 3 × 4 min washes in ice-cold 50 mM Tris-HCl and a dip in distilled water. Slides were then dried under a stream of cold air, desiccated overnight, and apposed to X-ray film (X-omat AR; Eastman Kodak) with standard 14C microscales (American Radiolabeled Chemicals, St. Louis, MO) for 9 days. Although 125I-FK 33,824 has been demonstrated to label multiple forms of μ-opioid binding sites (Rothman et al., 1987), under the present conditions the radioligand has been shown to label predominantly the high-affinity state of the μ-opioid receptor (Wong et al., 1994).

**Dopamine D1 Receptors.** Dopamine D1 receptor autoradiography was performed as described previously (Scibilia et al., 1992). In summary, tissue sections were allowed to warm to room temperature and then incubated for 30 min in Tris-HCl buffer (50 mM, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, and 1 mM MgCl2), 0.05 nM 125I-SCH 23982 (2200 Ci/mmol), and ketanserin (50 nM), which was included to prevent binding to 5-hydroxytryptamine2A receptors. Nonspecific binding was determined in the presence of 10 μM SKF 77434. After incubation, slides were subjected to 2 × 5-min washes in fresh ice-cold assay buffer and a dip in ice-cold distilled water. Slides were then dried under a gentle stream of cool air, desiccated overnight, and then apposed to X-ray film (X-omat AR;
Dopamine D2 Receptors. 125I-NCQ 298 was prepared from its des-iodo derivative NCQ 634 by the chloramine-T iodination technique (Lawrence et al., 1995). Autoradiographic techniques were carried out as described previously (Lawrence et al., 1995) to examine dopamine D2 receptor binding. In short, tissue sections were allowed to warm to room temperature and then preincubated (30 min, room temperature) in Tris-HCl buffer [170 mM, pH 7.6, containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 0.001% (w/v) ascorbic acid]. The sections were then incubated with 0.5 nM 125I-NCQ 298 (2000 Ci/mmol) for 60 min at room temperature in fresh buffer. Non-specific binding was defined as that remaining in the presence of 10 μM raclopride. The slide-mounted sections were then washed in ice-cold Tris-HCl buffer (170 mM, pH 7.6, 4 × 2 min) and finally rinsed in ice-cold distilled water (2 × 30 s). After washing, sections were dried under a gentle stream of cool air, desiccated overnight, and apposed to X-ray film (X-omat AR; Eastman Kodak) with standard 14C microscales (American Radiolabeled Chemicals) overnight, and apposed to X-ray film (X-omat AR; Eastman Kodak) with standard 14C microscales (American Radiolabeled Chemicals) for 24 h to visualize binding in the amygdala and mesencephalon. Sections were then reapposed to new film for 24 h to visualize binding in the amygdala and mesencephalon. All films used for autoradiography were developed automatically with a 100 Plus Automatic X-ray film processor.

Cellular Localization Using Nuclear Emulsion. To determine the precise cellular localization of dopamine D2 and D3 receptor binding, selected sections were apposed to coverslips that had been dipped in nuclear emulsion (LM1; Amersham Biosciences UK, Ltd.). Coverslips were then developed manually under darkroom conditions. Coverslips were submerged in Kodak D19 (3 min), transferred to a stop solution (1 min), fixed in a 20% (v/v) solution of Hypam Rapid Paper and Film fixer (Ilford Imaging UK Ltd, Cheshire, UK) (3 min), washed in distilled water (1 min), and dried. Brain sections were then counterstained with 0.1% thionin for identification of specific nuclei with reference to a stereotaxic atlas (Paxinos and Watson, 1986).

Data Analysis. Autoradiographic images on developed films were subsequently quantified using the Scion Imaging system (PC version of NIH Image) by comparison of the optical densities of autoradiographic images with those of the standard 14C microscales (American Radiolabeled Chemicals). Autoradiogram images were captured under constant illumination using an XC-77CE CD videocamera (Sony, Tokyo, Japan) with an attached Micro-Nikkor 55-mm lens (Nikon, Tokyo, Japan). Brain regions were identified after microscopic examination of the tissue sections counterstained with 0.1% thionin with reference to a stereotaxic atlas (Paxinos and Watson, 1986).

Statistical Analysis. Drinking data were analyzed with a one-way ANOVA, except analysis of daily fluid intake before and after withdrawal between groups, which was analyzed with a two-way ANOVA. Binding density of a radioligand in any particular brain region was determined by densitometry, and the mean value from all sections of an individual region was calculated for each rat. These values were then used to compute a group mean for each region in each group. Differences in binding density were determined by a one-way ANOVA, with a Bonferroni correction for multiple comparisons using SigmaStat, and P values are quoted under Results. In the case of dopamine D2 receptor binding in the NAcc, the raw data failed normality and so a Kruskal-Wallis nonparametric ANOVA was performed with a Student-Newman-Keuls post hoc analysis.

In this case, H values are quoted under Results. All graphical representations of the data were created in GraphPad Prism (GraphPad Software, San Diego, CA). A significance level of P < 0.05 was used throughout.

Results

Ethanol Consumption. As can be seen from Table 1, rats were found to have a mean preference of 86% for ethanol with mean ethanol consumption of 5.6 g/kg/day (n = 25). No significant difference was found for ethanol consumption between the five groups. Daily fluid intake for the ethanol naive control group was found to be 138 ml/kg/day compared with 164 ml/kg/day for the rats on free-choice ethanol (mean of the five groups). A significant difference was found between water consumption for the ethanol naive group compared with the free-choice ethanol groups: 138 and 24 ml/kg/day (mean of the five groups), respectively (Table 1). Interestingly, daily fluid intake increased with the number of days after withdrawal; the 10-day withdrawal group having essentially the same water intake to that of the ethanol naive group (136 compared with 138 ml/kg). Furthermore, withdrawal apparently had no effect on eating habits because rats continued to gain weight after ethanol withdrawal.

<table>
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<th>TABLE 1</th>
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<td>Comparison of ethanol and water consumption in FH rats, before and after ethanol withdrawal</td>
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<td>The overall data relating to daily fluid consumption of each of the six groups: ethanol naive (control), chronic ethanol consumption (0-day withdrawal) and the 1-, 2-, 5-, and 10-day withdrawal groups. Data relating to prewithdrawal are taken as daily fluid intake over a 5-week period. Data are expressed as the mean ± S.E.M., with n = 5 rats/group.</td>
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| Ethanol Naive | Prewithdrawal (Days Withdrawal) |
| --- |
| 0 | 1 | 2 | 5 |

| Weight (g) | Start | 270 ± 13 | 275 ± 8 | 281 ± 8 | 230 ± 11 | 226 ± 14 | 216 ± 16 |
| --- |
| End | 344 ± 7 | 348 ± 13 | 345 ± 6 | 321 ± 10 | 330 ± 18 | 310 ± 11 |

Δ Weight (g)

| Water intake (ml/kg/day) | 138 ± 11 | 21 ± 7b | 15 ± 3a | 17 ± 3a | 35 ± 12b | 32 ± 10a |
| --- |
| Ethanol (g/kg/day) | 15 ± 0 | 15 ± 0 | 16 ± 0 | 15 ± 0 | 16 ± 0 | 15 ± 0 |
| Preference for ethanol (%) | 87 ± 4 | 91 ± 1 | 90 ± 2 | 79 ± 3 | 85 ± 5 |

| Ethanol Naive | Postwithdrawal |
| --- |
| 0 | 1 | 2 | 5 |

| Weight (g) | Start | 345 ± 6 | 321 ± 10 | 330 ± 18 | 310 ± 11 |
| --- |
| End | 346 ± 6 | 325 ± 11 | 339 ± 16 | 336 ± 9 |

Δ Weight (g)

| Daily fluid intake (water) (ml/kg/day) | 108 ± 3a | 127 ± 6b | 118 ± 12b | 136 ± 12b |

a Significantly different to ethanol naive FH rats [F(5,24) = 41.1, P < 0.01].
b Daily fluid intake (milliliters per kilogram per day) postwithdrawal significantly different to prewithdrawal [F(3,22) = 64.3, P < 0.01].

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Effect of Chronic Ethanol and Withdrawal on μ-Opioid Receptor Binding. Autoradiographic detection of μ-opioid receptors by $^{125}$I-FK 33,824 was specific and fully displaceable by the opioid receptor antagonist naloxone (Fig. 1, A and B). Specific binding was detected throughout the forebrain and the mesencephalon. Compared with FH rats that had access to water only, FH rats that had access to a 5% ethanol solution and water showed elevated μ-opioid receptor density in most regions examined, although statistical significance was only reached in the hippocampus with a $+34\%$ increase in binding [$F(5,23) = 4.83, P < 0.05$] (Fig. 2F). Various effects were observed in the withdrawal groups compared with the ethanol naive and chronic groups in a number of brain regions examined (Fig. 2). Although neither chronic ethanol consumption nor withdrawal had any impact on $^{125}$I-FK 33,824 binding in the vertical limb of the diagonal band of Broca or thalamus (data not shown), in the NAcc, significant differences were found between the ethanol naive and days 1 and 10 withdrawal groups with increases in μ-opioid receptor binding of $+37$ and $+41\%$, respectively [$F(5,23) = 2.72, P < 0.05$]. In the lateral amygdala (Fig. 2D), binding in all withdrawal groups was found to be significantly different from the FH rats that received water only, and also from the chronic ethanol rats [$F(5,22) = 9.09, P < 0.05$]. These ranged from increases of $+31$ to $+39\%$ compared with the chronic ethanol group, and $+53$ to $+62\%$ increases compared with the ethanol naive group (Fig. 2D). Other regions with significant differences that were found between the withdrawal groups and the naive ethanol group included the cortex [$F(5,23) = 4.46, P < 0.05$], CPu [$F(5,23) = 3.7, P < 0.05$], mammillothalamic tract [$F(5,23) = 6.19, P < 0.05$], interpeduncular nucleus (data not shown), and the VTA.

Fig. 1. Representative autoradiogram of $^{125}$I-FK 33,824 binding to μ-opioid receptors in the rat brain (A) and of nonspecific binding as determined by the presence of 10 μM naloxone in FH rat brain (B). Representative autoradiogram of $^{125}$I-SCH 23982 binding to dopamine D$_1$ receptors restricted to the nucleus accumbens; caudate putamen; substantia nigra, pars reticulata; and entopeduncular nucleus (C) and dark-field image revealing binding of dopamine D$_1$ receptors throughout the substantia nigra, pars reticulata (E). Representative autoradiogram of $^{125}$I-NCQ 298 binding to dopamine D$_2$ receptors in the olfactory tubercle; caudate putamen; nucleus accumbens; central nucleus of the amygdala; subthalamic nucleus; substantia nigra, pars lateralis and pars compacta; and the ventral tegmental area (D) and dark-field image revealing binding of dopamine D$_2$ receptors throughout the substantia nigra, pars compacta (F). Scale bar for autoradiograms A to D, 2 mm; for photomicrographs E and F, 0.5 mm.
[\( F(5,22) = 6.2, P < 0.05 \) (Fig. 2)]. Overall, significant differences between the chronic ethanol and ethanol withdrawal groups were only found in the lateral and basolateral amygdala (Fig. 2, D and E).

**Effect of Chronic Ethanol and Withdrawal on Dopa- mine D1 Receptor Binding.** The binding of \(^{125}\text{I}-\text{SCH 23982}\) was essentially restricted to the NAcc; CPu; substantia nigra, pars reticulata (SNr); and the entopeduncular nucleus (EP) as shown in Fig. 1C. Specific binding was estimated to be 95 to 100% of the total binding because sections representing nonspecific binding were too faint to quantify. As can be seen from Fig. 3, no differences were observed in dopamine D1 receptor binding in the NAcc or CPu under these conditions. However, differences between the ethanol naive and 5- and 10-day withdrawal groups were observed in the SNr. This included a +34% increase in binding for the 5-day withdrawal group compared with control, and +31% increase for the day 10 withdrawal group \([F(5,24) = 3.54, P < 0.05]\). Increases in binding were also observed in the EP between the 5-day withdrawal group and both the naive and chronic ethanol groups (+35 and +33%, respectively) \([F(5,22) = 2.93, P < 0.05]\).

**Effect of Chronic Ethanol and Withdrawal on Dopa- mine D2 Receptor Binding.** Autoradiographic detection of

\[ \text{Density (dpm/mm}^2\text{)} \]

### Fig. 2. Effect of chronic ethanol consumption and subsequent withdrawal on the density of \(^{125}\text{I}-\text{FK 33,824 (0.1 nM)}\) binding to \(\mu\)-opioid receptors in the cortex (A), nucleus accumbens (B), caudate putamen (C), lateral amygdala (D), basolateral amygdala (E), hippocampus (F), mammillothalamic tract (G), and ventral tegmental area (H) in FH rat brain. The data are expressed as disintegrations per minute per square millimeter (mean ± S.E.M.) with \(n = 5\) rats/group, four sections per rat. All comparisons by one-way ANOVA, \(P < 0.05\), with a Bonferroni correction for multiple comparisons.

\* significantly different to ethanol naive FH rats.
\† significantly different to the FH rats in the chronic ethanol consumption group (0-day withdrawal). Naive, ethanol naive; chronic, chronic ethanol; d1w, day 1 withdrawal; d2w, day 2 withdrawal; d3w, day 3 withdrawal; d4w, day 4 withdrawal; d5w, day 5 withdrawal group; d10w, day 10 withdrawal.
dopamine D₂ receptors by ¹²⁵I-NCQ 298 was specific (Fig. 1D) and fully displaceable by the selective antagonist raclopride, with sections representing nonspecific binding being too faint to quantify. Binding of ¹²⁵I-NCQ 298 was located throughout regions of the FH rat brain, including the olfactory tubercle, CPU, NAcc, vertical limb of the diagonal band of Broca, central nucleus of the amygdala, VTA, and both the pars lateralis and pars compacta of the substantia nigra (SNc). Dopamine D₂ receptor densities were largely unaffected by either chronic ethanol consumption or withdrawal (Fig. 4). However, significant differences were found in the NAcc with an increase in binding observed for the 10-day withdrawal group compared with ethanol naive (+26%), and the 10-day withdrawal group compared with the chronic ethanol consumption group (+30%) \( [H = 11.6, df = 5, P < 0.05] \). In addition, dopamine D₂ receptor density was increased (+68%) in the 2-day withdrawal group compared with the control in the SNc \( [P(5,24) = 2.66, P < 0.05] \) (Fig. 4G).

**Discussion**

This study has examined the effect of chronic ethanol consumption and withdrawal on \( \mu \)-opioid receptor and dopamine D₁ and D₂ receptor density in the FH rat brain. Collectively, results obtained in this study suggest changes in \( \mu \)-opioid and dopamine receptor density after chronic consumption of ethanol and subsequent withdrawal may be reflective of different withdrawal states, and confirm that these systems are susceptible to modulation by ethanol.

**Effect of Chronic Ethanol on \( \mu \)-Opioid Receptor Density**

Increases of \( \mu \)-opioid binding after chronic ethanol consumption were observed in the NAcc (+21%) and CPU (+18%) compared with naive rats; however, these values did not quite reach statistical significance in the present study. Interestingly, we have previously documented very similar effects of ethanol consumption on \( \mu \)-opioid binding in FH rats (NAcc, +22%; CPU, +13%) that were significant (Cowen et al., 1999). These differences may reflect variations between individual animals, analysis of coronal (Cowen et al., 1999) versus horizontal (present study) sections, or differences in paradigms between the two studies because the former study was examining the effect of ethanol consumption during an alcohol deprivation effect induced by a period of abstinence. Whatever the difference, it is clear that ethanol consumption consistently causes an \( \sim \)20 to 25% increase in \( \mu \)-opioid binding in the FH NAcc.

A significant increase of \( \mu \)-opioid receptor density was observed in the hippocampus after chronic ethanol consumption. The hippocampus is involved in learning and memory processing (Simmons and Chavkin, 1996) but, as this and other studies (Lovinger et al., 1989) demonstrate, may also have a role in chronic ethanol consumption and withdrawal. Past studies are consistent with this suggestion with differences in hippocampal met-enkephalin tissue concentration being found between ethanol preferring rats and nonpreferring rats (Froehlich et al., 1987). Furthermore, it has been shown that hippocampal enkephalin systems are largely unaffected by chronic ethanol administration immediately after cessation of ethanol treatment (Lindholm et al., 2000); however, after 5 days of withdrawal from ethanol, a decrease in met-enkephalin tissue concentration was observed in the hippocampus (Lindholm et al., 2000). These data, and the fact that the NAcc also receives afferents from the hippocampus (Koob, 1999), suggest that this region may play a role in ethanol-seeking behavior and withdrawal.

**Effect of Ethanol Withdrawal on \( \mu \)-Opioid Receptor Density**

This study is the first to consider the effect of withdrawal after chronic ethanol consumption on \( \mu \)-opioid receptor density using autoradiography in alcohol-preferring FH rats. Interestingly, \( \mu \)-opioid receptor density tended to elevate after withdrawal and remained elevated over the time course of the study. Significant increases were observed in key regions involved in the mesolimbic pathway, including the VTA, NAcc, lateral amygdala, and basolateral amygdala. This finding suggests that adaptation after withdrawal can occur, leading to prolonged sensitization or responsiveness of the opioid system. Perhaps the most significant effect was that observed in the lateral amygdala with all withdrawal...
groups having significant increases in binding compared with their nondrinking counterparts and also the chronic ethanol consumption group. Many studies have provided evidence for a role of the amygdala in drug addiction (Koob and Nestler, 1997; Koob, 1999), and this study has confirmed that amygdaloid neurons are sensitive to chronic ethanol consumption and withdrawal. Although the mechanisms underlying these neurochemical changes are unknown, a possible explanation is a role in mediating negative emotional states such as anxiety, a symptom that has been associated with ethanol withdrawal.

In a previous study, we examined the effect of chronic ethanol consumption and acute withdrawal on μ-opioid receptor agonist-stimulated binding of [35S]GTPγS, as an index of possible downstream effects of ethanol on opioid receptors (Chen and Lawrence, 2000). Chronic ethanol consumption resulted in a decreased ability of the μ-opioid receptor agonist [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin to stimulate [35S]GTPγS binding in the NAcc and CPu, while not effecting binding in the amygdala; however, acute withdrawal resulted in an increase in [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin-stimulated [35S]GTPγS within the amygdala compared with chronic ethanol and a parallel recovery of binding in the NAcc and CPu (Chen and Lawrence, 2000). The present data go one step further and demonstrate that perturbations in μ-opioid receptors within the amygdala continue for at least 10 days after withdrawal. Taken together with the present findings, these data suggest that μ-opioid receptors in the amygdala are particularly sensitive to ethanol consumption and withdrawal. Importantly, the anticraving effect of nal-
trexone in FH rats is short-lived, with tolerance developing within a week of treatment, which is associated with a 3- to 4-fold up-regulation of μ-opioid receptors in the amygdala, an effect that is in relative terms greater than that observed in many other brain regions (Cowen et al., 1999). As such, the amygdala may represent an important locus for the targeting of novel therapeutics to reduce craving and assist in breaking the cycle of alcohol (and other substance) abuse.

Although the present data are suggestive of increases in μ-opioid receptor density within specific brain regions after chronic ethanol consumption and/or withdrawal, it is important to note that unequivocal confirmation that receptor density is increased would require saturation binding, particularly because an agonist radioligand has been used that may discriminate between different conformational states of the receptors. This is especially pertinent when one considers the effect of acute withdrawal on μ-opioid receptor-G protein coupling (Chen and Lawrence, 2000). Thus, it is equally possible based on the current data that ethanol withdrawal increases the proportion of μ-opioid receptors in a high agonist-affinity confirmation that is reflected as an increased binding density under the present conditions. It must however be remembered that the effects of withdrawal are region-specific, and not a global effect on all μ-opioid receptors.

Recently, μ-opioid receptor knockout mice were shown to be aversive to ethanol with no self-administration of ethanol under several experimental conditions (Roberts et al., 2000). Densities of μ-receptors in the alcohol preferring alko alcohol rat are also higher relative to their nonpreferring alko non-alcohol rat counterparts in the NAcc and VTA (De Waele et al., 1995; Marinelli et al., 2000) and similarly, alcohol-prefering rats have been shown to have a greater number of μ-opioid receptors in the NAcc and amygdala than alcohol nonpreferring rats (McBride et al., 1998). Results from these previous studies and the present study demonstrate that μ-opioid receptors are implicated in ethanol consumption and possibly withdrawal; however, the exact mechanisms for these effects remain elusive.

**Effect of Chronic Ethanol and Withdrawal on Dopamine D1 and D2 Receptor Binding.** This study demonstrated an increase in D2 receptor density in the 10-day withdrawal group compared with the ethanol naive. This finding is of interest because previous studies have suggested that the dopamine D2 receptors are resistant to modulation by chronic ethanol (Cowen and Lawrence, 2001). Taken together, these findings would suggest that dopamine D2 receptors in the NAcc are more susceptible to neuroadaptation after withdrawal rather than during chronic ethanol consumption. Chronic ethanol consumption has been associated with a significant increase in the Bmax for D2 receptors in the nucleus accumbens (Tajuddin and Druse, 1996). Previous reports in the literature have suggested that presynaptic dopamine D2 receptors in the NAcc have an autoinhibitory role, regulating dopamine release (Meador-Woodruff et al., 1991). Ethanol withdrawal has been associated with a decrease in dopamine release in the NAcc (Diana et al., 1993), although because 125I-NCQ 298 does not differentiate between pre- and postsynaptic receptors, it is difficult to conclude whether the increase in D2 receptor density is due to an increased density of presynaptic autoreceptors (which would decrease release) or postsynaptic receptors (due to decreased release) localized on enkephalin-containing efferents to the ventral pallidum. Studies examining changes in mRNA encoding dopamine D2 receptors in the VTA and NAcc would help to further address this issue.

Changes were also observed in D1 receptor density in terminal regions of the SNr and EP. Ethanol consumption reduces the firing rate of SNr γ-aminobutyric acid neurons (Diana et al., 1993), and thus increases in SNr receptor density after chronic ethanol withdrawal may occur via compensatory mechanisms that become activated after exposure and withdrawal from ethanol. Overall, these data indicate that both the dopamine D1 and D2 receptors play a role in ethanol consumption and withdrawal; however, due to the inconclusive and contradictory reports in this area (Hamdi and Prasad, 1993), further research is warranted. Whatever the case, this study suggests that both dopamine D1 and D2 receptors are sensitive to perturbation after withdrawal from chronic ethanol consumption. Moreover, because significant changes were found in the later stages of withdrawal, this perhaps suggests that counteradaptive neurochemical events within the NAcc are taking place and that the effect of ethanol withdrawal on these systems is long-lasting or delayed.

In conclusion, this study has examined the effect of chronic ethanol consumption and withdrawal on μ-opioid and dopamine D1 and D2 receptor density in the FH rat. Differences found between the ethanol naive, and chronic ethanol and withdrawal groups demonstrate that the opioid and dopamine systems are susceptible to modulation by chronic ethanol consumption and withdrawal in the FH rat. The exact mechanisms as to how these neurochemical changes occur require clarification and whether these changes reflect withdrawal states awaits confirmation.

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**References**


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