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ORIGINAL INVESTIGATION

F.S. Hall · I. Sora · G.R. Uhl

Ethanol consumption and reward are decreased in μ-opiate receptor knockout mice

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Abstract *Rationale:* Differences in μ-opiate receptor (MOR) gene expression may modulate the rewarding effects of ethanol. *Objective:* The effects of MOR gene knockout (KO) were examined in wild-type (+/+), heterozygote MOR KO (+/–), and homozygote MOR KO (–/–) mice on voluntary ethanol consumption, conditioned place preference produced by ethanol, and locomotor responses to ethanol in separate groups of mice. *Methods:* Voluntary ethanol consumption (2–32% v/v) was examined in a two-bottle home-cage consumption test. The conditioned place preference paradigm was a biased design. Mice received four pairings of ethanol (2.0 g/kg IP) on the initially preferred side and four pairings on the initially non-preferred side with saline. The difference in time spent on the initially non-preferred side (pre- versus post-conditioning) was the measure of drug-induced preference. After habituation to a novel locomotor test chamber mice were tested, on subsequent sessions, for ethanol induced locomotion (0.0, 0.5, 1.0, and 2.0 g/kg IP). *Results:* Heterozygous and homozygous MOR KO mice consumed less ethanol than wild-type mice. These effects appeared to be greater in female KO mice than in male KO mice. MOR KO mice, especially females, exhibited less ethanol reward in a conditioned place preference paradigm. These effects on ethanol reward were produced by reductions in MOR expression levels as small as 50%. MOR KO mice exhibited less ethanol-stimulated locomotion than did wild-type mice, an effect that was also largest in females. *Conclusions:* These data fit with the reported therapeutic efficacy of MOR antagonists in the treatment of human alcoholism. Allelic variants that confer differing levels of MOR expression could provide different degrees of risk for alcoholism.

Keywords Transgenic mice · μ-Opiate receptor · Ethanol · Reward · Conditioned place preference

Current NIDA regulations prevent G.R.U. from listing his affiliation with Johns Hopkins University School of Medicine

F.S. Hall (✉) · I. Sora · G.R. Uhl
Molecular Neurobiology Branch,
National Institute on Drug Abuse, Intramural Research Program,
5500 Nathan Shock Drive, Baltimore, MD 21224, USA
e-mail: Shall@intra.nida.nih.gov
Tel.: +1-410-550-2128, Fax: +1-410-550-2846

Introduction

Opiate antagonists can reduce human ethanol consumption (Davidson et al. 1999) and may be efficacious in the treatment of alcoholism (Berg et al. 1996; Kranzler et al. 1997, 1998b; Mason et al. 1999). The mechanisms for these effects are uncertain, however. It has been suggested that opiate antagonists could augment the sedative effects of ethanol and thus reduce its reinforcing properties (King et al. 1997; Swift et al. 1994), especially craving and cue-elicited positive reinforcement (Davidson et al. 1999; Palfai et al. 1999). These effects have been postulated to be more prominent in alcoholics, who manifest reduced basal opioid peptide and increased opiate receptor densities (Wand et al. 1998).

Studies in rodents have supported the idea that brain opioid systems can contribute to the rewarding effects of ethanol (for review see Reid et al. 1996). Animal models document the abilities of opiate antagonists to reduce ethanol consumption. Opiate antagonists that reduce ethanol consumption in rodents include naloxone (Beaman et al. 1984; Hubbell et al. 1986, 1988a; Hunter et al. 1984; Hyytia and Sinclair 1993; Myers and Critcher 1982; Petry 1995; Reid and Hunter 1984; Reid et al. 1991; Samson and Doyle 1985), naltrexone (Cowen et al. 1999; Davidson and Amit 1997a, b; Franck et al. 1998; June et al. 1998; Le et al. 1993; Myers and Lankford 1996; Nestby et al. 1999; Stromberg et al. 1998a, b; Volpicelli et al. 1986), nalmefene (June et al. 1998), LY117413 (Hubbell et al. 1988b), and MR2266 (Volpicelli et al. 1986). The specific μ-opiate receptor (MOR) antagonists β-funaltrexamine (Stromberg et al. 1998a) and CTOP (Hyytia 1993) also reduce ethanol consumption in some but not all studies (Franck et al.

1998; Le et al. 1993). Opiate antagonist effects are nearly always observed in continuous access paradigms, while repeated antagonist treatments appear necessary to demonstrate effects in limited access paradigms (Bienkowski et al. 1999).

MOR agonists such as morphine (Choi et al. 1990; Czirr et al. 1987; Hubbell et al. 1986, 1987, 1988b, 1993; Linseman and Harding 1990; Reid and Hunter 1984; Reid et al. 1991; Wild and Reid 1990) and fentanyl (Czirr et al. 1987) can increase ethanol consumption. These effects can occur without general changes in consummatory behavior (Petry 1995; Samson and Doyle 1985; but see Weiss et al. 1990). Low doses appear to be selective for reductions in ethanol self-administration while higher doses can produce more generalized decrements in consummatory behavior.

Naloxone and the selective MOR antagonist β-flunaltrexamine block development of ethanol-induced conditioned place preferences (Biala and Langwinski 1996; Matsuzawa et al. 1998, 1999). The selective δ-opioid receptor (DOR) antagonist naltrindole also blocks development of ethanol conditioned place preferences (Matsuzawa et al. 1998, 1999), while the κ-opioid receptor (KOR) antagonist nor-binaltorphimine is ineffective (Matsuzawa et al. 1998, 1999). Subthreshold doses of either the MOR agonist morphine or the DOR agonist TAN-67 produce conditioned place preferences in combination with subthreshold doses of ethanol (Matsuzawa et al. 1998). Indeed, although specific MOR antagonists reduce ethanol consumption, the specific DOR antagonists naltrindole and ICI 174864 also attenuate ethanol consumption (Franck et al. 1998; Krishnan-Sarin et al. 1995a, b; Le et al. 1993; but see also Hyytia 1993; Stromberg et al. 1998a). Ethanol-stimulated dopamine release in putative nucleus accumbens reward circuits is also attenuated by naltrindole (Acquas et al. 1993). Opioid antagonists could even be efficacious in treating alcoholism without playing such large roles in normal ethanol consumption. Since it thus remains to be seen how modulation of MOR alone attenuates ethanol consumption, the present experiments examined ethanol consumption and reward in wild-type and MOR KO mice.

MOR KO mice have previously been shown to be subsensitive to the analgesic effects of the MOR agonists morphine (Fuchs et al. 1999; Matthes et al. 1996; Sora et al. 1997b, 1999), heroin (Kitanaka et al. 1998), and morphine-6β-glucoronide (Kitanaka et al. 1998). They can also manifest reduced responses to delta agents including SNC80 (Sora et al. 1999), [D-Pen^2, D-Pen^5]-enkephalin (Fuchs et al. 1999; Qiu et al. 2000; Sora et al. 1997a), and deltorphin II (Matthes et al. 1998; Qiu et al. 2000) despite normal levels of DOR expression. By contrast κ agonists, including U50,488 and U69,593, produce normal analgesic effects in MOR KO mice (Fuchs et al. 1999; Sora et al. 1999). Spinal and supraspinal nociception is increased in MOR KO mice (Sora et al. 1997b) although chemical nociception is reduced (Sora et al. 1999).

Rewarding effects of morphine are also attenuated in homozygous MOR KO mice (Matthes et al. 1996; Sora et al. 2000). Interestingly, heterozygous MOR KO mice can even exhibit greater conditioned place preferences for morphine, indicating profound neural adaptations in these mice (Sora et al. 2000). The rewarding effects of many other drugs of abuse, including ethanol, have not been examined in these mice, however. Therefore, the effects of deletion of MOR on oral ethanol self-administration, ethanol conditioned place preference, and ethanol-stimulated locomotion were examined in this report.

Materials and methods

Subjects: μ-opiate receptor KO mice

Wild-type, heterozygous, and homozygous MOR KO mice were generated from random heterozygote crosses of mice developed in our laboratory maintaining a mixed C57/129sv background (Sora et al. 1997a, b). Mice were weaned at 21 days of age, and housed with same-sex littermates for the duration of the experiments, except where noted. Standard colony conditions were used: 24°C, 50% relative humidity, and ad libitum food and water according to AALAC guidelines. Experimentation began at between 8 and 12 weeks of age.

Experiment 1: voluntary ethanol consumption

Experimentally na male and female mice (n=10 per genotype gender) were housed singly beginning 1 week prior to the experiments. Water, food, and ethanol consumption were monitored in home cages. Initially, only food and water were available to determine baseline consumption. Subsequently, the subjects were given access to food, water, and ethanol in a standard two-bottle home-cage consumption paradigm. Fluids were made available in 50 ml polypropylene centrifuge tubes capped with rubber stoppers and standard sipper tubes. Fluid bottles and food were weighed every 2–3 days and consumption calculated in g/day, ml/day, and g/kg per day. The initial ethanol concentration was 2% and concentrations were increased every 2–3 days in the following progression: 2%, 4%, 8%, 12%, 16%, 24%, and 32%. The positions of the bottles were switched each time the bottles were changed. Food, water bottles, and ethanol bottles were weighed to determine the amount consumed. Mice were weighed once per week.

Experiment 2: conditioned place preference for ethanol

Conditioned place preference was assessed in experimentally na male and female mice (n=9–10 per genotype/gender). The apparatus consisted of a Plexiglas box divided into two chambers. During preference testing the two sides of the chamber (36×18×18 cm) were separated by a sliding Plexiglas divider with a 5-cm opening at the floor of the chamber. A solid divider was used during conditioning. One side of the apparatus had a wire floor, the other side corn cob bedding. The duration spent on each side of the apparatus was monitored using an Optivarimax animal activity monitoring apparatus (Columbus Instruments). After determination of initial preference, conditioning was conducted by confining the mice to one of the two sides with the solid Plexiglas divider. The subjects received four pairings of ethanol (2.0 g/kg IP) with the initially non-preferred side, and four pairings of saline with the initially preferred side. Subsequently the mice were tested again for side preference. The difference in time between the second preference test and the first preference test was used as the index of conditioned place preference.

Experiment 3: ethanol-stimulated locomotion

Baseline levels of activity were assessed in experimentally na male and female mice (n=9–10 per genotype/gender) in Plexiglas chambers (36×18×18 cm) where activity was monitored for 2 h using the Optivarimax system. On subsequent days, the mice were returned to this chamber for 1 h, after which they received injections of 0.0, 0.5, 1.0, or 2.0 g/kg ethanol i.p. (20% v/v). All subjects received all doses and each test session was separated by at least 48 h from the previous session. However, only the highest dose of ethanol produced any locomotor stimulation in this strain, so only the data from the 2.0 g/kg dose are presented.

Data analysis

Data were analyzed by ANOVA with the between-subjects measure of GENOTYPE and GENDER. Consumption data were analyzed as grams ethanol per kilogram body weight per day (g/kg per day). The within-subjects factor of CONCENTRATION was used for this data. Although food and water consumption was measured throughout, only the baseline data are presented, expressed in g/kg per day and ml/kg per day, respectively. For locomotor data the within-subjects factor of TIME was used. Post hoc analyses were performed using Fisher's PLSD.

Results

Experiment 1: voluntary ethanol consumption

There were no baseline differences between genotype groups in weight [F(2,27)=1.08, n.s.], food consumption [F(2,27)=1.59, n.s.], or water consumption [F(2,27)= 2.81, n.s.]. Similarly, there were no effects of gender on food or water consumption, although females weighed less than males [F(1,27)=22.00, P<0.001].

Wild-type mice consumed more ethanol than either heterozygous or homozygous KO mice. The effect of genotype was most manifest at low ethanol concentrations and only significant in females. Hence there was a significant GENOTYPE×GENDER×CONCENTRATION interaction [F(6,324)=1.88, P<0.05]. Based on these results, separate ANOVA tests were conducted for male and female mice. There were no significant differences in ethanol consumption between genotypes in male mice [F(2,27)=0.02, n.s.; Fig. 1A]. However, female wild-type mice consumed more ethanol than either heterozygote or homozygote KO female mice [F(2,27)=4.78, P<0.02; Fig. 1B].

Experiment 2: ethanol conditioned place preference

Ethanol produced greater conditioned place preference in female mice than male mice. Effects of GENOTYPE were thus dependent on GENDER. Ethanol produced a robust place preference in +/+ female mice that was absent from heterozygous or homozygous female MOR KO mice [Fig. 2; F(2,22)=4.2, p<0.03]. A similar pattern was observable in male mice, but less conditioned place preference was evident and the differences between phenotypes were thus not statistically significant [F(2,27)=1.6, n.s.].

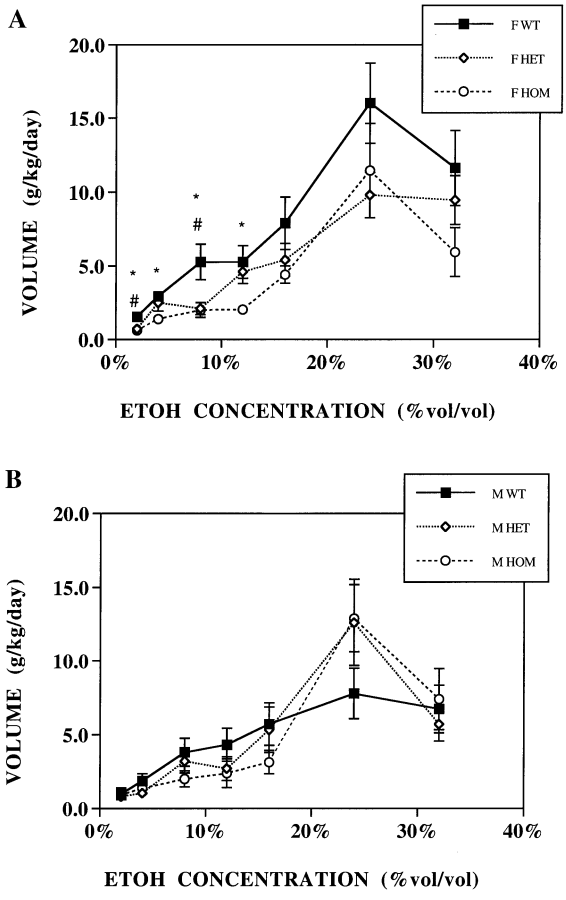

Fig. 1A,B Voluntary ethanol consumption. The data represent voluntary consumption of ethanol in grams per kilogram of body weight per day (g/kg per day), for female (**A**) and male (**B**) +/+, +/–, and –/– MOR knockout (KO) mice. The data are expressed as mean ± standard error of the mean. * Significant post hoc difference using Fisher's PLSD, +/+ vs –/–, P<0.05. # Significant post hoc difference using Fisher's PLSD, +/+ vs +/–, P<0.05. *FWT* Female wild-type, *FHET* female heterozygous, *FHOM* female homozygous, *MWT* male wild-type, *MHET* male heterozygous, *MHOM* male homozygous

Experiment 3: ethanol-stimulated locomotor activity

There were no statistically significant differences in baseline locomotor activity in male mice, although homozygous males tended to have greater activity [GENOTYPE: F(2,270=2.8, P<0.08]. Heterozygous and homozygous females had greater baseline activities than wild-type mice at the beginning of the locomotor testing sessions [GENOTYPE×TIME: F(22,297)=2.0, P<0.01; Fig. 3]. There were no differences in locomotion at the end of the test after activity had habituated.

Ethanol-stimulated locomotion was analyzed compared to activity after saline injection (Fig. 4). The 2 mg/kg dose of ethanol modestly increased locomotion in these subjects [ethanol: F(1,54)=5.9, P<0.02]. When data from each gender are pooled, the trend toward greater ethanol-stimulated activity in wild-type than in KO mice does not reach statistical significance, however.

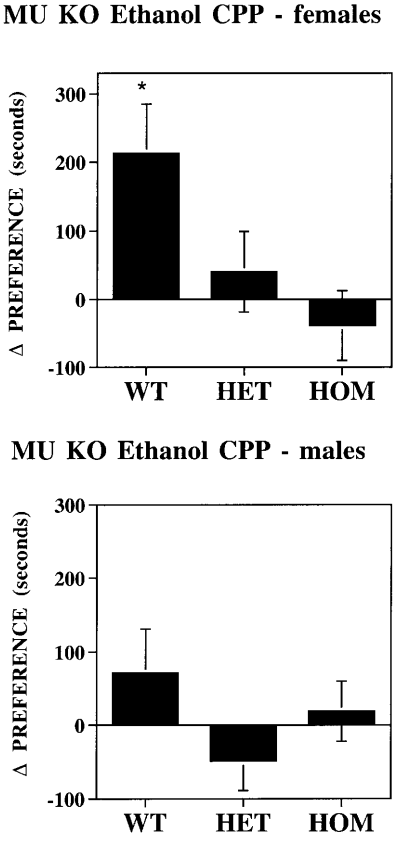

Fig. 2 Conditioned place preference (*CPP*). The data represent the difference between the initial preference and the postconditioning preference for the initially non-preferred side in +/+, +/–, and –/– MOR KO mice. The data are expressed as mean ± standard error of the mean. * Significant post hoc difference using Fisher's PLSD, +/+ vs –/–, $P<0.05$

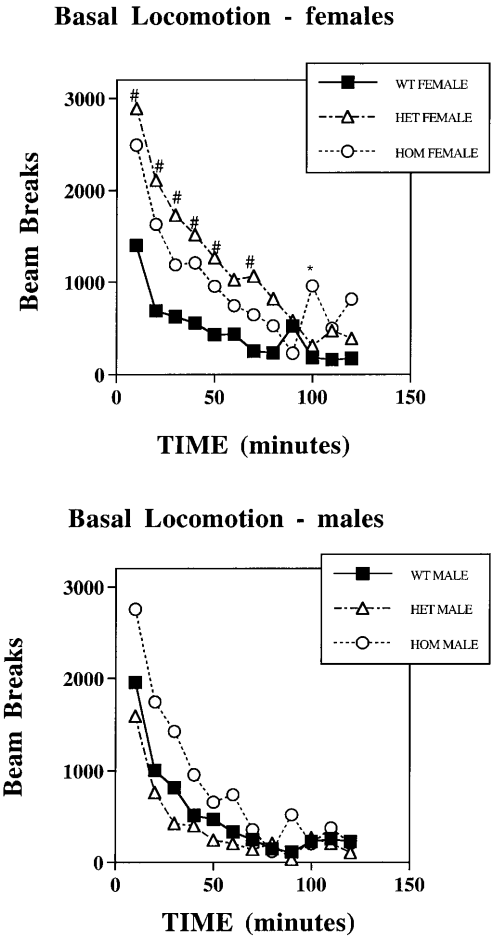

Fig. 3 The data represent baseline locomotor activity in male and female +/+, +/–, and –/– MOR KO mice. The data are expressed as mean ± standard error of the mean. * Significant post hoc difference using Fisher's PLSD, +/+ vs –/–, $P<0.05$. # Significant post hoc difference using Fisher's PLSD, +/+ vs +/–, $P<0.05$

Discussion

Deletion of the MOR in mice substantially reduces responses to ethanol, including the rewarding effects of ethanol assessed in both self-administration and conditioned place preference paradigms. Complete deletion of the MOR is not necessary to produce this attenuation of ethanol reward. The effect of MOR deletion is just as robust in heterozygous mice, which exhibit 50% of wild-type levels of MOR expression (Sora et al. 1997b), as it is in homozygous mice devoid of MOR expression. These data strongly suggest that alterations in MOR levels can be important determinants of ethanol self-administration and potentially predisposition to alcohol abuse. The data also accord with the idea that treatments that reduce MOR levels or block MOR receptors can have therapeutic efficacy for alcoholism.

Our observations that ethanol consumption and ethanol place preference are attenuated in MOR KO mice are congruous with previous literature which has shown that MOR antagonists decrease voluntary ethanol consumption in rodents (Beaman et al. 1984; Cowen et al. 1999; Davidson and Amit 1997a, b; Franck et al. 1998;

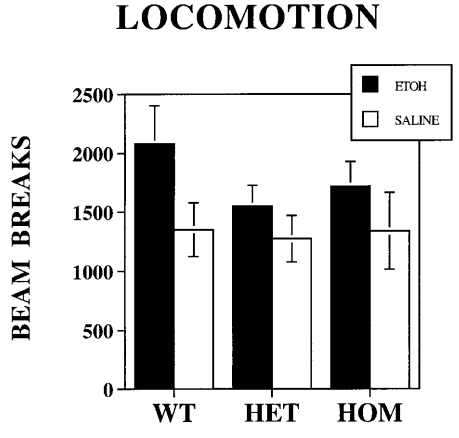

Fig. 4 The data represent ethanol-stimulated locomotor activity (2.0 g/kg IP) in male and female +/+, +/–, and –/– MOR KO mice. The data are expressed as mean ± standard error of the mean. There were no significant differences

Hubbell et al. 1986, 1988a; Hunter et al. 1984; Hyytia and Sinclair 1993; June et al. 1998; Le et al. 1993; Myers and Critcher 1982; Myers and Lankford 1996; Nestby et al. 1999; Petry 1995; Reid and Hunter 1984; Reid et al. 1991; Samson and Doyle 1985; Stromberg et al. 1998a, b; Volpicelli et al. 1986). Although most of these studies have been done in rats, opiate antagonists can also reduce ethanol consumption in mice (Phillips et al. 1997). Reductions in ethanol consumption in MOR KO mice affirm a role of MOR in ethanol self-administration in mice. These data are consistent with the ability of μ-opiate antagonists to attenuate ethanol-induced dopamine release in nucleus accumbens, where dopamine action has been tied to rewarding effects (Benjamin et al. 1993; Tanda and Di Chiara 1998). Other more indirect evidence, taken together with the present results, also points toward opiate/ethanol interactions. Rats selectively bred for low ethanol consumption express lower levels of MOR, particularly in terminal regions of the mesolimbic dopamine system, than rats bred for high consumption (McBride et al. 1998; Soini et al. 1999; de Waele et al. 1995). Ethanol-preferring rats exhibit more robust ethanol-stimulation of higher-activity hypothalamic β-endorphins than ethanol-avoiding rats (de Waele and Gianoulakis 1994). Injections of antisense oligonucleotides targeted to MOR mRNA also reduce ethanol intake (Myers and Robinson 1999). Effects of MOR KO on ethanol consumption and reward do not exclude possible roles for other opiate receptor subtypes as well, however. In addition to the pharmacological evidence for DOR involvement noted above, DOR levels are higher in mouse strains that consume much ethanol (de Waele and Gianoulakis 1997), perhaps as a consequence of decreased endogenous opioid levels (George et al. 1991).

Previous pharmacological studies in this area have used antagonist drugs that often lack optimal specificity. These limitations contrast with those of MOR KO mice. Conversely, developmental neuroadaptations in other systems to lifelong loss of functional MORs may account for alterations in responses to ethanol. However, there is little evidence for such adaptations in these mice. For instance, although it might be speculated that developmental alterations in other opiate receptors might underlie these effects, no alterations in either DOR or KOR have been observed in MOR KO mice (Sora et al. 1997b). Furthermore, our recent studies using expression chips have documented only a limited number of other genes whose expression is altered significantly in whole brain mRNA prepared from these KO mice (Liu et al. 1999). The paucity of data indicating major developmental alterations in other neurotransmitter systems in MOR KO mice, along with the convergence of KO and antagonist data, provide substantial support for the idea that MOR changes, in themselves, are likely to be responsible for the reduced ethanol consumption and reward.

Reductions in ethanol reward appeared to be highly gender-dependent. However, since the male mice in this study demonstrated much smaller ethanol responses, particularly for conditioned place preference, it cannot be concluded that deletion of MOR has greater effects on female mice. Pharmacological antagonism of MOR certainly does have effects in males. Indeed, the vast majority of studies demonstrating reduced ethanol consumption after opiate antagonist treatment have been conducted in male subjects, and no studies, to the knowledge of these authors, have examined the effects of gender on opiate antagonist reduction of voluntary ethanol consumption. Nonetheless, as found in the present experiments voluntary ethanol consumption, under continuous access conditions, is greater in females (Meliska et al. 1995; Nocjar et al. 1999; Phillips et al. 1997). This may be in part because estradiol and progesterone can modulate MOR binding (Carter and Soliman 1998). Thus, it remains possible that deletion of MOR has greater effects in female mice, but the issue cannot be resolved based on the present data. These experiments are currently being replicated in MOR KO mice that have been placed on a C57Bl/6 background, which is more sensitive to the rewarding effects of ethanol and may help resolve this issue.

Nevertheless, since MOR KO mice display reduced ethanol consumption, it might be supposed that allelic MOR variants, especially those that decrease MOR expression or function, might reduce risks of alcoholism. Examination of the initial human MOR polymorphic markers in alcoholics or polysubstance abusers has produced a mixed picture (Bergen et al. 1997; Kranzler et al. 1998a; Sander et al. 1998; Smolka et al. 1999). However, increased human MOR levels, assessed by postmortem [^{3}H]-naloxone binding, are found in alcoholics compared to non-alcoholics (Ritchie and Noble 1996). Also, sons of alcohol-dependent men exhibit greater ACTH responses to naloxone (Wand et al. 1999). Since the present data indicate that the level of MOR expression could have a substantial impact on alcohol consumption and even vulnerability alcoholism, improved elucidation of the human MOR locus and definition of the variants that actually contribute to altered levels of expression of this receptor will be important. Conceivably such data could help determine how genetic, and other factors that modulate levels of MOR expression, affect sensitivity to alcohol reward and addiction in humans.

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