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μ-Opioid Receptor Knockout Mice Do Not Self-Administer Alcohol[1,2]

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ABSTRACT

Opioid peptides long have been hypothesized to play a role in ethanol reinforcement. Neuropharmacological studies have shown that opioid receptor antagonists decrease ethanol self-administration in rodents and prevent relapse in humans. However, the exact mechanism for such powerful effects has remained elusive. The availability of μ-opioid receptor knockout mice has made possible the direct examination of the role of the μ-opioid receptor in mediating ethanol self-administration. In the present experiments, both nosepoke and lever operant ethanol self-administration and several tests of two bottle-choice ethanol drinking were studied in these genetically engineered mice. In no case did knockout mice show evidence of ethanol self-administration, and, in fact, these mice showed evidence of an aversion to ethanol under several experimental conditions. These data provide new evidence for a critical role for μ-opioid receptors in ethanol self-administration assessed with a variety of behavioral paradigms and new insights into the neuropharmacological basis for ethanol reinforcement.

Opioid peptides acting via the μ-opioid receptor have been implicated in the reinforcing effects of ethanol (Froehlich, 1995; Ulm et al., 1995; Herz, 1997). Evidence for this implication has come from both correlational and pharmacological studies. In the correlational approach, opioid peptide levels and opioid receptor levels are compared between strains or lines of rodents differing in their ethanol intake. μ-Opioid receptor densities have been shown to be higher in ethanol-preferring strains in brain regions such as the extended amygdala postulated to mediate the rewarding effects of drugs of abuse. For example, densities of μ-receptors were higher in the alcohol-preferring AA rats relative to their nonpreferring ANA counterparts in the nucleus accumbens (shell region) and ventral tegmental area (de Waele et al., 1995; Soini et al., 1999). The alcohol-preferring P rats had a greater number of μ-opioid recognition sites in the nucleus accumbens and amygdaloid nuclei than alcohol-nonpreferring NP rats (McBride et al., 1998). Furthermore, alcohol-accepting C57BL/6J mice showed higher levels of μ-receptor labeling in the amygdala than the alcohol-avoiding DBA/2J mice (de Waele and Gianoulakis, 1997). These results suggest a link between μ-receptor binding potential (greater receptor densities and/or higher binding affinities) and alcohol preference.

Naloxone and naltrexone, nonselective opioid receptor antagonists, have been shown to reduce ethanol consumption in humans (O'Malley et al., 1992; Volpicelli et al., 1992) and animals (Ulm et al., 1995; Rodefer et al., 1999). The growing availability of more selective opiate receptor antagonists has allowed researchers to examine more specifically the role of μ-opioid receptors in ethanol consumption with a pharmacological approach. For example, the μ-receptor antagonist β-funaltrexamine decreased ethanol drinking in genetically heterogeneous Wistar rats (Stromberg et al., 1998) and a rat line selectively bred for high alcohol drinking (Froehlich, 1995). Ethanol consumption by selectively bred AA rats was reduced by administration of the μ-receptor antagonists CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, a somatostatin analog; Hyytïa, 1993) and naloxonazine (Honkanen et al., 1996). Thus, these data provide pharmacological evidence for a role of μ-opioid receptors in ethanol-drinking behavior.

The creation of new models such as transgenic and null mutant mice with recombinant DNA technology has provided a third approach with which to study the role of specific receptors in mediating the reinforcing effects of ethanol (Wehner and Bowers, 1995). This approach has particular promise for elucidating the mechanism of action for the re-

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ABBREVIATIONS: WT, wild-type; KO, knockout; FR, fixed ratio.

inforcing effects of ethanol because ethanol acts on many different neurochemical systems, and vulnerability to alcoholism has been hypothesized to result from polygenic influences. Several laboratories have produced μ-opioid receptor null mutants that show decreased sensitivity to various effects of morphine (Kieffer, 1999). For example, these mice display decreased morphine-induced analgesia, attenuated morphine withdrawal symptoms, and a lack of conditioned place preference to morphine (Matthes et al., 1996). These animals provide a means to investigate the role of the μ-opioid receptor in the actions of ethanol in a manner that complements and extends the traditional pharmacological and correlational approaches (Gold, 1996).

The purpose of this study was to test the hypothesis that the μ-opioid receptor is important for the reinforcing effects of ethanol with μ-opioid receptor null mutant mice and their wild-type (WT) counterparts. Ethanol self-administration was examined with several different approaches because it has been suggested that hypothesis testing in genetically engineered mice should focus on at least three well validated tasks within the particular behavioral domain of interest (Crawley, 1998). A single test of ethanol consumption can be ambiguous, and characterizing a strain as an "alcohol avoider" requires corroborating data from a number of tasks related to ethanol consumption. Therefore, in this study operant ethanol self-administration was studied with multiple approaches: two different operant procedures and several tests of two bottle-choice ethanol consumption.

Materials and Methods

Subjects

The generation of μ-opioid receptor knockout (KO) mice has been described previously (Matthes et al., 1996). Briefly, gene inactivation was obtained by disruption of the second exon of the μ-opioid receptor gene in 129/Sv embryonic stem cells. Germline transmission occurred from the breeding of chimeric animals with C57/BL6 mice. Mice heterogeneous for the mutation were obtained on a 50% 129/50% C57/BL6 genetic background and used as founder animals to produce the F1 animals used in these experiments. A total of 20 male homozygous μ-opioid receptor KO and 20 WT mice imported from Strasbourg, France, were used in these experiments. Mice were housed one to three per cage in a temperature-controlled room in which the lights were on a 12-h light/dark cycle with lights off at 10:00 AM. Mice were 5 to 6 months of age at the initiation of experiments, except for the final two bottle-choice test in which mice were ~11 months of age. Three KO mice and one WT mouse were not included in the experiments because of death or signs of illness (weight loss and lethargy) before the initiation of the experiments.

Operant Apparati and Training

Six operant testing chambers were outfitted for nosepoke responding and six were outfitted for lever responding. Each of these 12 chambers measured 14.9 × 15.2 × 18.3 cm and was housed within larger exterior boxes (Coleman coolers) equipped with exhaust fans serving to ventilate the chambers and to mask background noise. One wall of each nosepoke operant chamber was equipped with two small holes (0.9 cm in diameter; 4.2 cm apart; 1.5 cm from the grid floor) with adjacent photocells to detect nosepoke responses. Between the nosepoke holes there was a slot through which a food trough connected to a feeder, or two plastic drinking cups separated by a divider (7.5 × 10 cm), was inserted. One wall of each lever operant chamber was equipped with two levers (2.5 cm in width; 5 cm apart; 2.5 cm from the grid floor). A lever press required 5 ± 1 g of downward force and resulted in the disruption of a photocell beam. Food or fluid delivery and recording of operant responses (photocell beam breaks) were controlled by a microcomputer.

For food-reinforced operant training, only the nosepoke boxes were used and testing was performed as detailed in Heyser et al. (1997). Mice were tested in daily 15-min sessions conducted 5 days a week for 3 weeks. For the duration of these operant food sessions, mice were maintained under conditions of food restriction (21 h). Animals were weighed before testing and given food for 3 h after completion of the session. On each weekend day, mice received food for 3 h. Mice were initially trained to respond for food on a fixed ratio 1 (FR1) schedule of reinforcement for 5 days. One nosepoke in the active hole resulted in the delivery of a food pellet (20 mg; P. J. Noyes Co. Inc., Lancaster, NH). The FR was increased to 3 for another 5 days and to 5 for the final 5 days. The location of the active nosepoke hole remained constant and no discriminative cues (explicit stimuli) other than the sound of the feeder mechanism were associated with food delivery.

Specific details of the ethanol self-administration training used in experiments 1 and 2 are described below. Ethanol dilutions (5, 8, and 10% w/v) were made up with 95% ethyl alcohol and water. Sodium saccharin (Sigma Chemical Co., St. Louis, MO) was added to water or the ethanol solutions to achieve 0.2% (w/v), and sucrose (Fisher Scientific, Pittsburgh, PA) was added to water to achieve a final concentration of 20% (w/v). Regardless of whether the operant was a nosepoke or a lever press, a continuous reinforcement schedule was used (FR1), resulting in the delivery of 0.01 ml of fluid into one of the two drinking cups. Mice were tested in daily 30-min sessions, 5 days/week.

For two bottle-choice tests, mice were singly housed, and a bottle containing 10% ethanol and one containing water were placed on each cage. The positions of the tubes on the cage were random and ~6 in. apart. Mice were allowed free choice of these drinking solutions for 24-h periods with simultaneous free access to food. Ethanol intake was calculated based on bottle weights before and after each 24-h period, and body weights were used to calculate grams per kilogram ethanol consumed.

Experiment 1, Nosepoke Operant: Food, Ethanol, and Sucrose. Seven KO and eight WT mice were used in this experiment. Phase 1 involved the examination of food-reinforced responding as described above. The purpose of this experiment was to determine whether there were global differences between KO and WT mice in motivated behavior and/or learning before examining operant ethanol self-administration behavior. In phase 2, a saccharin-fading procedure adapted from one used in rats (Roberts et al., 1998) was used to attempt to establish ethanol as a reinforcer in the nosepoke boxes. Water bottles were removed from the mouse cages 2 h before operant sessions. For the first 6 days of training, one hole was blocked and nosepokes in the available hole were associated with the delivery of 0.2% saccharin. For the remainder of training, both holes were available and responding in one hole resulted in delivery of saccharin/ethanol and responding in the other resulted in delivery of water. The progression of saccharin-fading training was as follows: 4 days of saccharin versus water, 6 days of 5% ethanol + saccharin versus water, 3 days of 5% ethanol, 3 days of 8% ethanol + saccharin versus water, 3 days of 8% ethanol, and 14 days of 10% ethanol + saccharin versus water. The holes were alternated daily throughout this phase. For the next 16 days, 10% ethanol and water were available with the nosepoke hole associated with each kept constant.

The purpose of phase 3 was to determine whether the KO mice, which showed very low rates of responding for both ethanol and water, would self-administer an alternate liquid reinforcer (sucrose) in this operant oral self-administration paradigm. The water hole and ethanol hole were reversed, with responding in the previous water hole resulting in delivery of 20% sucrose and responding in the previous ethanol hole resulting in delivery of water. After 7 days, the holes were again reversed for 5 days to examine the ability of the mice to discriminate between the available reinforcers. To determine

whether the mice would self-administer ethanol + sucrose preferentially over sucrose alone, a final phase of 6 days of operant testing was undertaken. The holes were again reversed such that responding in the previous sucrose hole resulted in delivery of sucrose + 10% ethanol and responding in the previous water hole resulted in delivery of sucrose alone.

One week after completion of operant sessions, mice were tested for two bottle-choice (10% ethanol versus water) drinking. If ethanol was established as a reinforcer during operant conditioning, then mice would be expected to show a preference for ethanol under free-choice drinking conditions. If, however, ethanol was never established as a reinforcer during operant conditioning, then very low free-choice ethanol drinking would be expected. Mice were singly housed and a bottle containing 10% ethanol and one containing water were placed on each cage. The positions of the tubes on the cage were random and ~6 in. apart. Mice were allowed free choice of these drinking solutions for two 24-h periods with simultaneous free access to food. Ethanol intake was calculated based on bottle weights before and after each 24-h period and body weights were used to calculate grams per kilogram ethanol consumed.

Experiment 2: Two Bottle-Choice and Lever Operant. Six KO and 6 WT mice were used in this experiment. Because free-choice drinking in experiment 1 was conducted only after exposure to ethanol in the operant paradigm, one of the purposes of this experiment was to examine this behavior both before and after extensive exposure to ethanol. Therefore, the first phase of experiment 2 involved 3 days of single housing with constant access to two drinking tubes, randomly placed, of 10% ethanol and water. Mice and drinking bottles were weighed daily. While still singly housed, mice were subjected to a saccharin-fading procedure with drinking bottles to attempt to establish ethanol as a reinforcer before testing under operant conditions. The progression of training was as follows: 2 days of 0.2% saccharin versus water, 2 days of 5% ethanol + saccharin versus water, 2 days of 5% ethanol versus water, 2 days of 8% ethanol + saccharin versus water, 2 days of 8% ethanol versus water, 4 days of 10% ethanol + saccharin, and 8 days of 10% ethanol versus water.

At the conclusion of free-choice testing, mice were reintroduced to their cage mates, and except for two cases in which mice had to be separated, the mice adjusted well to the reestablishment of group housing conditions. Operant sessions involving a lever press operant were initiated with one lever associated with 10% ethanol and the other one associated with water. The levers were not alternated. For the first 3 days, the mice were restricted to 6 h of water after operant testing to increase the motivation of the mice to seek liquid reinforcement. Water was available ad libitum for the remaining 37 test days.

Immediately after completion of the final operant test, blood was sampled from each mouse for blood alcohol level determinations. Approximately 40 μl of blood was obtained by cutting 0.5 mm from the tip of each mouse's tail with a clean razor blade. Blood was collected in capillary tubes and emptied into Eppendorf tubes containing 1 μl of heparin (1000 U/ml) and kept on ice. Samples were centrifuged and serum was decanted into fresh Eppendorf tubes. The serum was extracted with trichloracetic acid and assayed for ethanol content with the NAD-alcohol dehydrogenase enzyme spectrophotometric method (Sigma Chemical Co.).

A final 3-day, two bottle-choice test between 10% ethanol and water was completed as a comparison to that performed at the beginning of this experiment. Again, mice and drinking bottles were weighed daily.

Experiment 3: Two Bottle-Choice Test. One of the potential caveats of these experiments concerns whether mice have consumed enough ethanol to experience its pharmacological effects. One could argue that to self-administer ethanol, sufficient ethanol must be consumed to experience these effects. Therefore the purpose of experiment 3 was to examine the effect of forced ethanol exposure on subsequent two bottle-choice drinking. Four KO and five WT mice were used in this experiment. Mice were singly housed and, along with food, received a single bottle containing 10% ethanol for 3 days. Bottles were weighed daily and the mice were weighed and observed carefully for signs of dehydration and/or illness. The mice then were allowed access to 10% ethanol and water in a two bottle-choice procedure for 3 days.

Statistical Analyses

Operant data (Figs. 1-4) were analyzed with ANOVAs with the between-subject factor group (KO versus WT) and the within-subject factors hole or lever (e.g., food hole versus inactive hole or ethanol versus water) and session. Significant interactions were investigated with simple effects analyses followed by post hoc Tukey's tests. Specific details of these analyses are included in *Results* for each experiment. The two bottle-choice parameters shown in Table 1 (ethanol and water consumption and preference ratios) were compared between WT and KO mice with Tukey's tests, as were blood alcohol levels and ethanol responding in grams per kilogram.

Results

Experiment 1, Nosepoke Operant: Food, Ethanol, and Sucrose. Figure 1 shows operant responding for food across the 5 days of FR1, FR3, and FR5 sessions. Data were analyzed with a three-way mixed design ANOVA with the between-subject factor strain and the within-subject factors nosepoke hole (food versus inactive) and session. Nosepoke responding was higher in the food-associated hole than the inactive hole ($F_{1,13} = 237.2$; $P < .001$) and there was an overall effect of session ($F_{14,182} = 15.3$, $P < .001$) and ses-

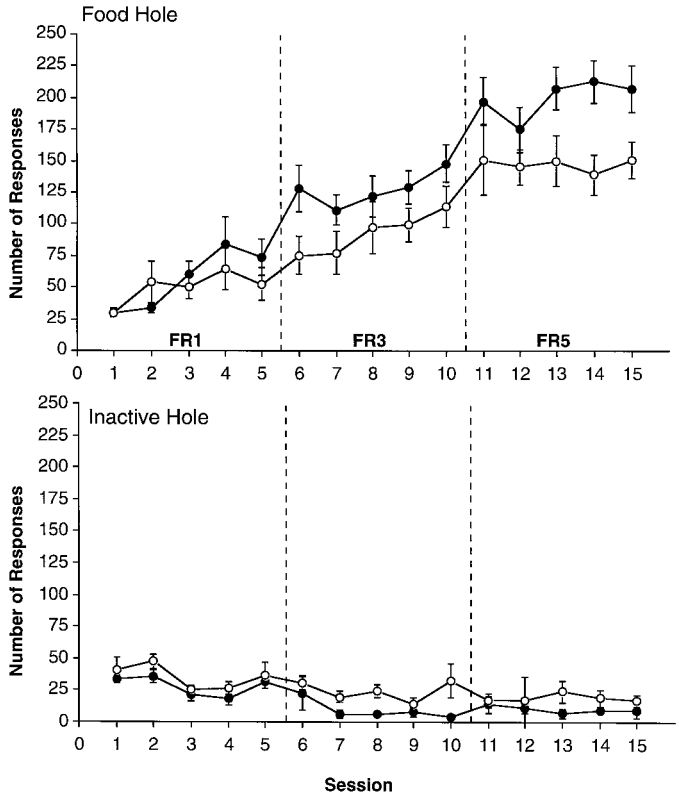

Fig. 1. Operant responding for food (nosepoke responding) in μ-opioid receptor KO mice (●) and their WT counterparts (○). The FR requirement for a single food pellet increased every 5 days. Overall, nosepoke responding was higher in the food-associated hole (top) than the inactive hole (bottom). The mice learned to perform the nosepoke operant for food and altered their response rates as the response requirement increased.

TABLE 1
Two bottle-choice tests from the three independent experimental phases
Data from the final day of each 3-day 24-hr two bottle-choice test is presented. Preference ratios were calculated as the ethanol consumption/total fluid consumption.

	Wild Type			Knockout		
	Ethanol	Water	Preference Ratio	Ethanol	Water	Preference Ratio
		ml			*ml*	
Experiment 1						
Postoperant	4.43 ± 0.39	1.05 ± 0.14	0.80 ± 0.04	1.50 ± 0.39[a]	4.39 ± 0.49[a]	0.26 ± 0.07[a]
Experiment 2						
Preoperant	2.32 ± 0.51	5.43 ± 1.06	0.31 ± 0.07	2.67 ± 0.34	4.08 ± 0.59	0.40 ± 0.05
Postoperant	3.10 ± 0.48	2.80 ± 0.62	0.54 ± 0.08	1.20 ± 0.29[a]	3.53 ± 0.34	0.25 ± 0.07[a]
Experiment 3						
Postforced ethanol	2.92 ± 0.41	2.44 ± 0.78	0.57 ± 0.10	1.15 ± 0.38[a]	5.05 ± 0.33[a]	0.18 ± 0.06[a]

[a] Denotes a significant difference between WT and KO mice ($P < .05$).

sion × nosepoke hole ($F_{14,182} = 32.1$ $P < .001$). This provides evidence that the mice learned the task in that they responded more in the active hole and altered their response rates relative to the FR requirement. There was no overall strain difference in operant responding, including both the food-associated nosepoke hole and the inactive hole ($P > .05$). There was, however, a strain × hole effect ($F_{1,13} = 12.4$; $P < .01$). Simple effects analysis of the interaction revealed that KO mice responded significantly more than WT mice for food ($P < .01$) and significantly less in the inactive hole. These data suggest that both strains learned to respond for food, with KO mice perhaps outperforming WT mice.

Figure 2 shows operant nosepoke responding for 10% ethanol (top) versus water (bottom) after the saccharin-fading procedure in the same mice used for food training. Data were analyzed with a three-way mixed design ANOVA with the between-subject factor strain and the within-subject factors nosepoke hole (ethanol versus water) and session. There was an overall difference between the strains ($F_{1,13} = 15.7$; $P < .01$), with WT mice responding significantly more than KO mice. There was no interaction between strain and hole. KO mice responded less for ethanol and also responded less in the water-associated hole.

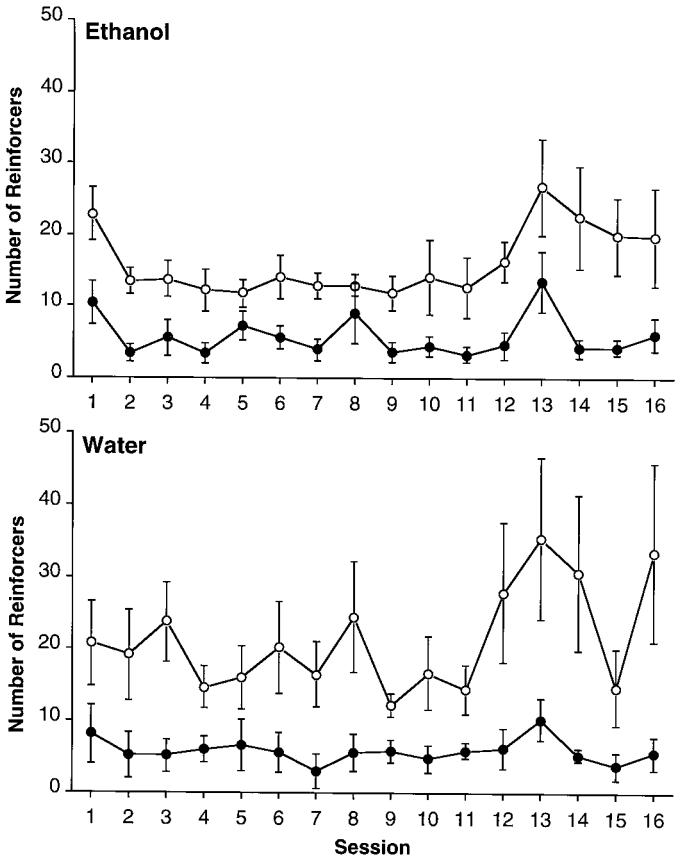

Fig. 2. Operant self-administration (nosepoke responding) of 10% ethanol (top) versus water (bottom) in KO (●) and WT (○) mice after a saccharin-fading procedure. There was a significant difference between the strains, with WT mice responding more for both ethanol and water than KO mice.

Data for these same mice allowed to operantly respond for sweetened solutions were analyzed by three-way mixed design ANOVA with the between-subject factor strain and the within-subject factors nosepoke hole (sucrose versus water and ethanol + sucrose versus sucrose) and session. When the animals were allowed to respond for the alternate liquid reinforcer, sucrose, the KO mice self-administered sucrose, although overall they responded less than WT mice ($F_{1,13} = 8.5$; $P < .01$); Fig. 3, left). There were also significant effects of hole ($F_{1,13} = 137.1$; $P < .001$), session ($F_{11,143} = 4.2$; $P < .001$), and hole × session ($F_{11,143} = 11.8$; $P < .001$), but no significant interaction involving strain. These data indicate that although KO mice did not respond as much as WT mice, they did respond preferentially for sucrose and switched response allocation when the holes were reversed. This suggests that KO mice are capable of acquiring and maintaining nosepoke behavior associated with liquid reinforcement. More importantly, however, when given a choice between ethanol + sucrose and sucrose alone, KO mice preferentially responded for sucrose, whereas WT mice responded equally for both solutions (Fig. 3, right). There were no effects of solution or session in WT mice, whereas KO mice responded significantly more for sucrose alone than ethanol + sucrose ($F_{1,6} = 5.8$; $P < .05$). There was a significant interaction between solution and session in this group ($F_{5,30} = 4.1$; $P < .01$) with a solution effect in sessions 16 to 18. These data suggest that although WT mice displayed no preference for one solution over the other, KO mice responded less to the hole associated with the ethanol-containing solution than the sucrose solution.

The mice used in the nosepoke experiment were tested for two bottle-choice (10% ethanol versus water) drinking after their operant experience (Table 1). KO mice consumed much less ethanol and more water than WT mice, resulting in significantly lower preference ratios in KO mice ($P < .05$).

Experiment 2: Two Bottle-Choice and Lever Operant. The final 16 days (of a total of 40) of lever pressing for

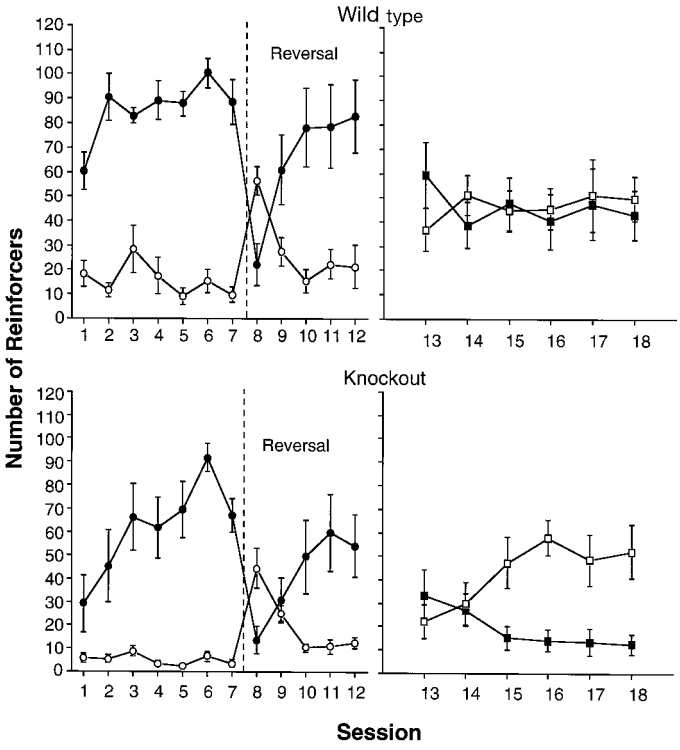

Fig. 3. Operant self-administration (nosepoke responding) for sucrose versus water (left) and ethanol + sucrose versus sucrose (right) in KO and WT mice. KO mice responded less than WT mice overall for sucrose and water. All mice responded for more sucrose than water and switched response allocation to continue to receive sucrose when the holes were reversed. Again, there was a strain difference, with KO mice responding significantly less than WT mice overall for ethanol + sucrose and sucrose. There was no significant effect of solution or session in WT mice, whereas KO mice responded for significantly less ethanol + sucrose than sucrose alone. ●, sucrose; ○, water; ■, ethanol + sucrose; □, sucrose.

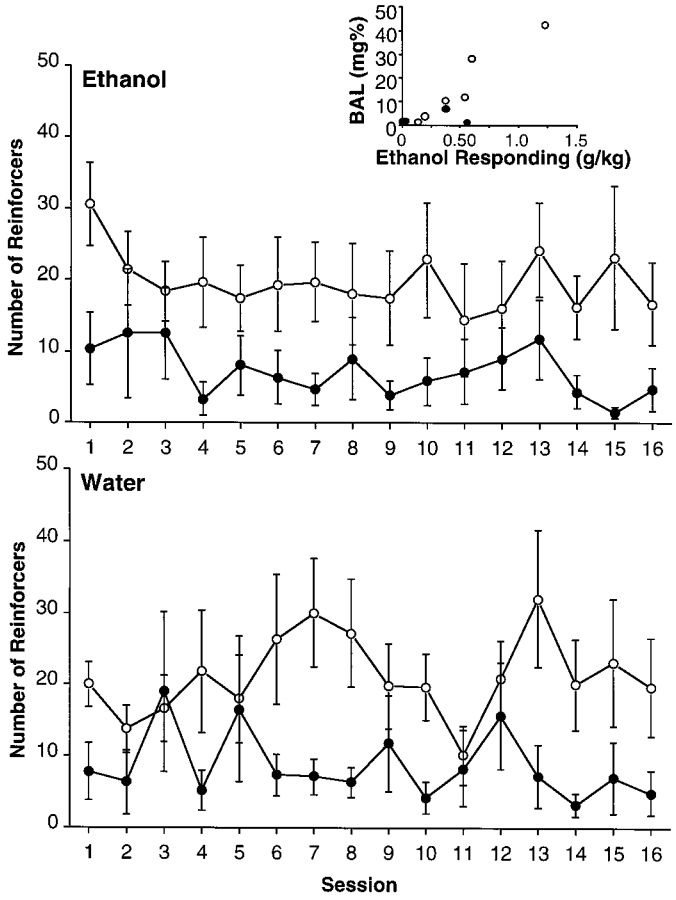

Fig. 4. Operant self-administration (lever pressing) of 10% ethanol (top) versus water (bottom) in KO (●) and WT (○) mice after a saccharin-fading procedure. The last 16 days of a total of 40 is shown. There was a significant overall strain difference, with higher responding in WT relative to KO mice. The lack of significant interactions involving strain suggests that neither strain responds differentially for ethanol and water. Blood alcohol levels were determined immediately after the final operant session and the inset to this figure depicts these values (milligrams per 100 milliliters) against ethanol responding relative to body weight (grams per kilogram). KO mice responded for significantly less ethanol relative to body weight than WT mice and had lower resulting blood alcohol levels.

10% ethanol are shown in Fig. 4. These data were analyzed with a three-way mixed design ANOVA with the between-subject factor strain and the within-subject factors lever (ethanol versus water) and session. There was a significant overall strain difference ($F_{1,10} = 4.5$; $P < .05$), with higher responding in WT compared with KO mice. The lack of significant interactions involving strain suggests that neither strain responds differentially for ethanol and water. Blood alcohol levels were determined immediately after the final operant session and the inset to Fig. 4 depicts these values (milligrams per 100 milliliters) compared with ethanol deliveries (grams per kilogram). KO mice responded for significantly less ethanol relative to body weight than WT mice ($P < .05$) and had lower resulting blood alcohol levels ($P < .05$). Although WT mice responded for ethanol, they also appeared to respond equivalently for water; however, KO mice responded less for both ethanol and water.

The mice used in this experiment were tested for two bottle-choice (10% ethanol versus water) drinking both before and after operant testing (Table 1). KO mice consumed much less ethanol and more water than WT mice after operant experience, resulting in significantly lower preference ratios in KO mice ($P < .05$). In contrast, this difference in ethanol preference between KO and WT mice was not present before lever operant testing.

Experiment 3: Two Bottle-Choice Test. The effect of forced ethanol exposure on two bottle-choice drinking was examined to determine whether the experience of the pharmacological effects of ethanol by KO mice would alter their subsequent preference for ethanol. There was no significant strain difference in consumption when ethanol was the only available fluid (KO, 4.9 ± 0.8 ml and WT, 5.3 ± 1.7 ml on the last day). The mice then were allowed access to 10% ethanol and water in a two bottle-choice procedure for 3 days. The results of the final test day of this experiment are shown in Table 1. In the free-choice portion of the experiment, KO mice consumed less ethanol and more water and therefore had significantly lower ethanol preference ratios than WT mice ($P < .05$). The results of this experiment suggest that even if KO mice had an experience with ethanol equal to WT mice, they subsequently consumed very little when given a choice.

Discussion

The overall finding in this set of studies is that mice lacking the μ-opioid receptor do not self-administer ethanol. This was true in two different operant procedures (nosepoke and lever), with sweetener added and in a two bottle-choice drink-

ing procedure (with or without prior ethanol experience). WT mice consumed ~0.6 g/kg ethanol in the 30-min operant tests and 10 to 13 g/kg ethanol in the 24-h bottle drinking tests. In contrast, KO mice consumed ~0.1 g/kg ethanol in the operant tests and 3 to 5 g/kg ethanol in the bottle drinking tests. The two bottle-choice ethanol consumptions of WT mice in the present study were comparable to those reported for C57BL/6J mice by Belknap et al. (1993) as well as in the congenic C57BL/6J WT littermates of D_2 dopamine receptor KO mice (Phillips et al., 1998). The consumption of ethanol by WT mice in the operant tests was lower than that reported in the related C57BL/6J inbred mouse strain (Elmer et al., 1987); however, the mild food restriction protocol used in this previously published study may have enhanced drinking. Overall, these results support the hypothesis that KO mice are capable of learning an operant task and respond for food and a sweet solution, but that they do not respond for ethanol in an operant task and consume only modest amounts in a two bottle-choice situation.

Ethanol consumption of KO mice was insignificant and similar to that observed in inbred mouse strains such as DBA/2J mice that are well acknowledged alcohol avoiders (Belknap et al., 1993; Risinger et al., 1998). KO mice were not, however, incapable of performing in an operant task because they consistently responded for food and sucrose. In fact, the same mice that were responding at high rates for food (Fig. 1) rapidly decreased their responding when ethanol was made available (Fig. 2), only to increase again when sucrose was substituted for ethanol (Fig. 3). This shows a strong tendency of these mice to alter their behavior in reference to ethanol as the reinforcer and suggests that ethanol does not maintain responding in KO mice.

An important strength of these experiments is that multiple approaches to examining ethanol consumption were used. Two bottle-choice drinking as the only measure of ethanol's reinforcing capacity is potentially confounded by palatability and lack of information regarding patterns of consumption (Cicero, 1979; Meisch, 1994), and it has been suggested that the operant self-administration technique provides a more reliable test of reinforcement (Meisch, 1994). Several laboratories have successfully developed operant ethanol self-administration procedures with C57BL/6 mice (Elmer et al., 1986; Risinger et al., 1998; Middaugh et al., 1999). In the present experiments, a two-manipulanda (two holes or two levers), limited-access approach was used in which the mice were not deprived of food or water and were trained with a variation of the sweetened solution-fading procedure first used in rats (Samson, 1986).

Despite vigorous responding for ethanol, WT mice did not show strong evidence of higher ethanol self-administration compared to responding for water (operant responding for ethanol was no higher than operant responding for water and responding for ethanol + sucrose was no different from responding for sucrose alone). However, an important observation was that these mice showed evidence that the ethanol was being consumed because the liquid receptacles were empty at the completion of sessions and there was little or no evidence of spillage in the bedding under the ethanol cups. This observation, combined with the results shown in Fig. 4 of detectable blood alcohol levels in self-administering WT mice, suggests that these mice were self-administering ethanol and not just performing the operant behaviors randomly. The question of why these mice showed selective responding in the hole associated with food and sucrose, but were less selective when ethanol was available remains unanswered. Higher rates of responding were maintained for food and sucrose; therefore, it is possible that the mice had less "free time" to perform at the alternate manipulandum. It is also possible that low-dose stimulant properties of ethanol, manifest in mice, increased behavior in a generalized fashion, and, because "extra" responding has no ill consequence, mice expended this energy nosepoking and lever pressing for water.

The background strains used in genetic engineering (in this case C57BL/6 × 129/Sv) are critically important and potentially problematic in studies characterizing the effects of the absence of a gene product on behavior (Wehner and Bowers, 1995; Gold, 1996). For example, 129/Sv mice often perform poorly in learning tasks and C57BL/6 mice are known for their ethanol-drinking behavior (Crawley and Paylor, 1997). The related 129/J mouse strain, although not avoiding ethanol, does not consume as much as C57BL/6 mice in two bottle-choice tasks (Belknap et al., 1993). Therefore the use of KO mice backcrossed onto single inbred strains (congenic mice) more thoroughly characterized for ethanol-related traits is a potential future approach. However, interactions between the gene of interest and strain-specific genes often can lead to unexpected phenotypes (Wehner et al., 1998).

The present results support a critical role for μ-opioid receptors in ethanol reinforcement. The exact mechanism by which ethanol modulates μ-receptor function is unclear, although several possibilities have been proposed. Acute and chronic ethanol administration is associated with increases in levels of the endogenous opioid β-endorphin (Ulm et al., 1995). Also relevant, moderate concentrations of ethanol (25–100 mM) have been shown to increase the binding capacity of μ-opioid receptors (Charness, 1989). Opioid receptors (both μ and δ), in turn, appear to mediate ethanol-induced stimulation of dopamine release (Di Chiara et al., 1996). The enhancement of dopamine by μ-agonists is thought to occur via presynaptic μ-receptors on dopaminergic cell bodies in the ventral tegmental area (Herz, 1997). Interestingly, dopamine D_2 receptor KO mice show very similar avoidance of ethanol in a two bottle-choice test as do the μ-KO mice tested presently (Phillips et al., 1998), suggesting that both μ-opioid and dopamine D_2 receptors are important in mediating the reinforcing effects of ethanol. How exactly ethanol modulates μ-receptor function will be a major challenge of future research. Nevertheless, the apparently critical role of the μ-receptor for ethanol reinforcement refocuses the neuropharmacology of ethanol reinforcement and opens a novel avenue for exploring the neuroadaptations associated with alcoholism.

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