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Ethanol-conditioned place preference is reduced in dopamine D2 receptor-deficient mice

Christopher L. Cunningham[a,e,*], MacKenzie A. Howard[a,e], Sylvia J. Gill[b], Marcelo Rubinstein[f], Malcolm J. Low[d,e], David K. Grandy[b,c,e]

[a]*Department of Behavioral Neuroscience, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA*
[b]*Department of Physiology and Pharmacology, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA*
[c]*Department of Cell and Developmental Biology, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA*
[d]*Vollum Institute, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA*
[e]*Portland Alcohol Research Center, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA*
[f]*Instituto de Investigaciones en Ingenieria Genetica y Biologia Molecular, CONICET, and Departmento de Biologia, University of Buenos Aires, Buenos Aires, Argentina*

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Abstract

Pharmacological blockade studies have supported a role of the dopamine system in ethanol reward for many years, but receptor subtype specificity has been difficult to establish. Recently, genetically engineered mice lacking functional dopamine D2 receptors have been shown to drink less ethanol in a two-bottle choice task. To determine whether reduced ethanol intake reflects a reduction in ethanol reward, D2 receptor-deficient [knockout (KO)] mice were compared to heterozygous (HET) and wild-type (WT; C57BL/6 × DBA/2 F2 hybrid) mice in a place conditioning task. Under conditions that produced reliable place preference in both WT and HET mice, KO mice showed no evidence of place conditioning, suggesting that D2 receptor gene inactivation reduced ethanol reward or the ability to learn about ethanol reward. Consistent with previous findings, this mutation also produced a gene dose-related reduction in basal activity levels. Moreover, KO and HET mice showed enhancement of ethanol-stimulated activity relative to WT mice. However, differences in basal and ethanol-stimulated activity did not explain the differences in place conditioning. Overall, this study strongly supports the conclusion that dopamine D2 receptors normally influence ethanol reward in mice.

Keywords: Ethanol; Conditioned place preference; Locomotor activity; Dopamine D2 receptor; Knockout mice; C57BL/6×DBA/2 F2 hybrid mice

Among the many neurobiological processes thought to contribute to the rewarding effects of abused drugs, activity within the mesolimbic dopamine system is probably implicated most frequently [2,15,29]. In the case of ethanol, oral self-administration has been found to stimulate dopamine release in the nucleus accumbens of rats (e.g., [18,31]), and a variety of dopamine receptor agonists and antagonists have been found to alter ethanol self-administration (e.g., [28,32]). Although such findings confirm a role for dopamine in ethanol self administration, progress toward a complete understanding of this role has been slowed by the paucity of antagonists with selectivity in vivo for each of the receptor subtypes within the two main families of dopamine receptors, the D1-like (D1 and D5) and the D2-like receptors (D2, D3 and D4).

However, the recent availability of dopamine receptor-deficient [knockout (KO)] mice has shed new light on the role played by the dopamine D2 receptor. More specifically, Phillips et al. [23] reported that 24-h ethanol intake and preference in a two-bottle (ethanol vs. water) choice procedure were reduced in mice lacking D2 receptors compared to normal wild-type (WT) or heterozygous (HET) siblings. KO mice showed aversions (preference ratios below 0.4) at all ethanol concentrations (3%, 6% or 10%), whereas WT and HET mice showed preference (ratios >0.5) at all concentrations, consistent with the pattern normally shown

* Corresponding author. Department of Behavioral Neuroscience, L470, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA. Tel.: +1-503-494-2018; fax: +1-503-494-6877.

E-mail address: cunningh@ohsu.edu (C.L. Cunningham).

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by the background strain (C57BL/6J). The authors interpreted their results as supporting the conclusion that dopamine D2 receptors are normally involved in determining sensitivity to ethanol's rewarding effects. The reduced ethanol intake observed in D2 receptor-deficient mice was assumed to reflect a *decrease* in ethanol reward because a critical target for ethanol-stimulated dopamine release was missing.

One of the difficulties in interpreting reduced consumption of ethanol is that lower intakes may actually result from an *increase* in ethanol reward. That is, KO mice may consume less ethanol because gene deletion has enhanced ethanol reward, thereby requiring less ethanol to achieve the same effect normally produced in WT mice. For example, rewarding effects that accompany ethanol-stimulated dopamine release might be greater because lack of D2 autoreceptors eliminates negative feedback mechanisms that usually limit dopamine release. Alternatively, ethanol reward might be greater because elimination of D2 receptors delays clearance of synaptic dopamine by its transporter. In fact, the latter possibility is strongly supported by a recent in vivo voltammetry study that found a lower volume of exogenous dopamine was required to produce a target electrochemical signal in dorsal striatum of D2 KO than in WT mice [16]. This study also reported a significantly lower dopamine clearance rate in KO mice, but no differences in basal or K^+-stimulated dopamine release or affinity/number of dopamine transporter binding sites. Thus, lower ethanol intake observed in D2 receptor-deficient mice could reflect greater ethanol reward caused by a decrease in dopamine transporter activity.

The present studies used a place conditioning task to determine whether dopamine D2 receptor deficiency produces an increase or decrease in ethanol reward. This task, which assesses an animal's tendency to approach or avoid environmental cues previously paired with drug [30], has been quite useful for studying ethanol reward in mice (e.g., [3,4,9,11,14]) and for detecting genetic differences in ethanol reward (e.g., [5,8,10,13]). Given the monotonic relationship between ethanol dose and conditioned place preference [13,27], it is assumed that factors that increase or decrease ethanol reward will produce increases or decreases in conditioned place preference, respectively. Thus, if ethanol reward is increased by elimination of D2 receptors, KO mice should show stronger ethanol-induced conditioned place preference. However, if loss of D2 receptors reduces ethanol reward, KO mice should display weaker conditioned preference.

1. Method

1.1. Subjects

Dopamine D2 receptor-deficient mice were originally generated by targeted mutagenesis in 129/SvPas embryonic stem cells injected into C57BL/6J (B6) blastocysts [19]. Subsequently, an incipient congenic strain was established by backcrossing mice that were HET for the D2 receptor gene mutation to WT B6 mice for five generations, at which time nonsibling heterozygotes were inbred to expand the colony [20]. Because ethanol-induced place conditioning is generally stronger in DBA/2 than in B6 mice [13], congenic B6 mice carrying the mutation were intercrossed with DBA/2 mice for two generations. F2 mice were then genotyped individually by Southern blotting or polymerase chain reaction to identify mice homozygous (KO), HET or WT for the D2 receptor mutation. Both male and female mice were tested. Ages on the first training day ranged from 71 to 134 days.

Mice were transferred from the breeding colony to the laboratory colony about 1 week before training began. Same sex and genotype mice were housed two to four per polycarbonate cage in a Thoren rack. Lab chow and water were continuously available in the home cages. All experimental procedures were conducted during the light phase of a 12:12 light/dark cycle (lights on at 07:00 h). Temperature in the lab remained at $21 \pm 1°C$ throughout the experiment. These studies were conducted in accord with the NIH "Principles of laboratory animal care."

1.2. Apparatus

The conditioning and testing apparatus consisted of 12 acrylic and aluminum boxes ($30 \times 15 \times 15$ cm). Each of these boxes was enclosed in a separate ventilated, light and sound-attenuating chamber (Coulbourn Model E10-20). General activity and position within the apparatus were recorded by computer using six sets of infrared photodetectors positioned at 5-cm intervals, 2.2 cm above the floor along the length of each box. The floors of each box consisted of interchangeable halves of two distinct textures. "Grid" floors were constructed from 2.3-mm stainless-steel rods mounted 6.4 mm apart on an acrylic frame. "Hole" floors were made from sheets of perforated stainless steel (16 GA) with 6.4-mm round holes on 9.5-mm staggered centers. The combination of grid and hole floor textures was selected based on previous studies showing that saline-treated control mice from many different genotypes spend approximately equal amounts of time on each floor type during preference tests (e.g., [8,13,14]). Floors and conditioning boxes were wiped with a damp sponge, and litter paper beneath the floors was changed between animals.

1.3. Procedure

To increase confidence in the reliability of our findings, two independent experiments were conducted using the same procedure. Experiment 1 tested only KO and WT mice, whereas Experiment 2 tested all three genotypes. These studies were run by the same experimenter about 3 months apart. Mice used in Experiment 2 (mean age = 119

Table 1
Number of mice assigned to each group

	Experiment 1		Experiment 2		
Genotype/group	Female	Male	Female	Male	Total
WT/GRID+	27[a]	20	9	7	63
WT/GRID−	28	20	13	7	68
WT/saline	6	6	–	–	12
KO/GRID+	16	16	10	6	48
KO/GRID−	17	15	8	5	45
KO/saline	5	6	–	–	11
HET/GRID+	–	–	10	9	19
HET/GRID−	–	–	10	9	19

[a] Due to equipment malfunction, preference test data from one subject in this group were lost.

days) were about 1 month older than mice used in Experiment 1 (mean age = 89 days). Each experiment involved one habituation session, eight conditioning sessions, and one test session. On the habituation day, all mice were weighed, injected with saline (12.5 ml/kg ip) and immediately exposed to the apparatus with a smooth paper floor for 5 min.

During the conditioning phase, approximately equal numbers of mice from each genotype and sex were randomly assigned to conditioning subgroups (GRID+ and GRID−) that differed in terms of which floor type served as CS+ (see Table 1 for subgroup sizes). On each 5-min conditioning trial, the apparatus contained only one floor type, and mice had access to the entire apparatus. On CS+ trials, mice in the GRID+ subgroups were injected intraperitoneally (ip) with ethanol [2 g/kg, 20% (v/v)] immediately before placement on the grid floor, whereas mice in the GRID− subgroups received ethanol paired with the hole floor. The 2-g/kg dose was expected to produce an intermediate level of place conditioning in WT mice, thereby providing an opportunity to see either a reduction or enhancement of place preference in KO mice. On CS− trials, each subgroup was exposed to the opposite floor type immediately after saline injection. Thus, this design matches subgroups for overall exposure to both floor types, ethanol and saline. Four trials of each type were given on alternating days in a counterbalanced order. A 2-day break separated the first four sessions from the second four sessions.

In Experiment 1, a subset of animals from each genotype was assigned to control groups that were exposed to both floors but received saline injections on all trials during the conditioning phase. These groups were included in order to determine whether genotypes differed in unconditioned preference for the floor textures.

The experiment concluded with a 30-min place preference test that began approximately 24 h after the last conditioning trial. The apparatus was configured with half-grid, half-hole floors, and relative position of the floors was counterbalanced within each subgroup. Mice were injected with saline and immediately placed in the center of the apparatus on the border between the two floors. Activity and amount of time spent on the grid floor were recorded during the test.

2. Results

Preliminary statistical analyses of preference test data from groups common to both experiments indicated no significant main effects or interactions involving experiment or sex. Therefore, in order to simplify presentation, data were collapsed over these variables in analyses reported below. All data were analyzed by analysis of variance (ANOVA). α-Level set at .05.

2.1. Conditioning trial activity

Mean activity rates during each 5-min ethanol (CS+) and saline (CS−) conditioning trial are depicted in Fig. 1. Ethanol produced an increase in activity relative to saline in all three genotypes. This effect diminished over trials except in HET. Activity on saline trials was directly related to frequency of the WT allele and decreased across trials in all three genotypes. Three-way ANOVA (Genotype × Drug × Trials) yielded significant main effects of Genotype [$F(2,259) = 8.0$, $P < .0005$], Drug [$F(1,259) = 411.3$, $P < .0001$] and Trials [$F(3,777) = 32.6$, $P < .0001$]. The Genotype × Drug [$F(2,259) = 8.8$, $P < .0002$] and Genotype × Trials [$F(6,777) = 4.2$, $P < .0005$] interactions were also significant. Separate analyses of the genotype effect for each drug suggested that the Genotype × Drug interaction was due to a significant main effect of Genotype on saline [$F(2,259) = 68.4$, $P < .0001$] but not on ethanol [$F(2,259) = 1.0$] trials. Pairwise comparisons of data averaged across all four saline trials indicated that all genotype differences were significant (Bonferroni-corrected P's < .001).

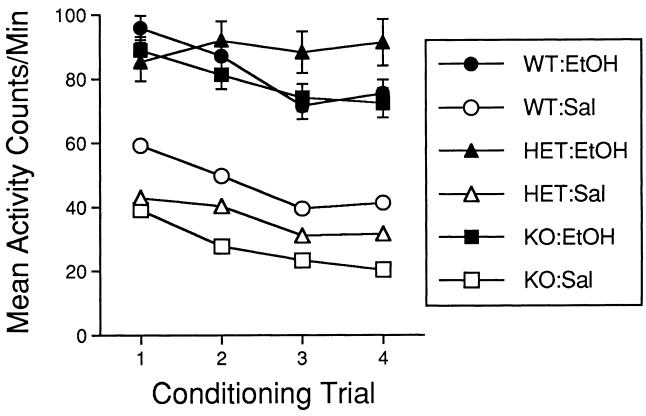

Fig. 1. Mean ± S.E.M. activity counts/min during each of the 5-min CS+ (EtOH = ethanol) and CS− (Sal = Saline) conditioning trials (collapsed across experiment and conditioning subgroup). The numbers of mice from each genotype were WT = 131, HET = 38 and KO = 93. Error bars for saline trials are hidden by the symbols.

Because strain differences in basal activity complicate interpretation of the ethanol-stimulated activity, ethanol data were also analyzed as the difference between activity on each CS+ and the corresponding CS − trials. This analysis indicated a consistently greater ethanol-induced activation in both KO and HET mice compared to WT (see Fig. 2). Genotype × Trials ANOVA revealed a significant main effect of Genotype [$F(2,259) = 8.8$, $P < .0002$], but no other effects. Pairwise comparisons of difference scores averaged across all four trials showed a significant difference between WT and each of the other genotypes (Bonferroni-corrected P's < .02), but no difference between KO and HET mice.

2.2. Preference test

Fig. 3 shows mean time spent on the grid floor by each conditioning subgroup during the final preference test. In this experimental design, evidence of place conditioning is provided by comparing the difference between the GRID+ and GRID − conditioning subgroups, which differed only in the floor–ethanol relationship [7]. As can be seen, WT and HET mice spent more time on the grid floor when it was paired with ethanol (GRID+) than when it was paired with saline (GRID −), reflecting development of conditioned place preference. In contrast, the two KO subgroups performed similarly, indicating absence of place conditioning. Unexpectedly, both KO subgroups showed a preference for the grid floor.

Two-way ANOVA (Genotype × Conditioning Subgroup) of the preference test data yielded significant main effects of Genotype [$F(2,255) = 10.8$, $P < .0001$] and Conditioning Subgroup [$F(1,255) = 17.4$, $P < .0001$], and a significant interaction [$F(2,255) = 7.1$, $P = .001$]. Follow-up comparisons between the GRID+ and GRID − subgroups within each genotype confirmed the presence of a significant conditioned place preference in WT and HET mice [Bonferroni-corrected P's < .005], but not in KO mice. It should be noted that the difference between KO and WT mice was also significant when each experiment was analyzed separately (interaction P's < .02) and thus did not depend on pooling data across experiments.

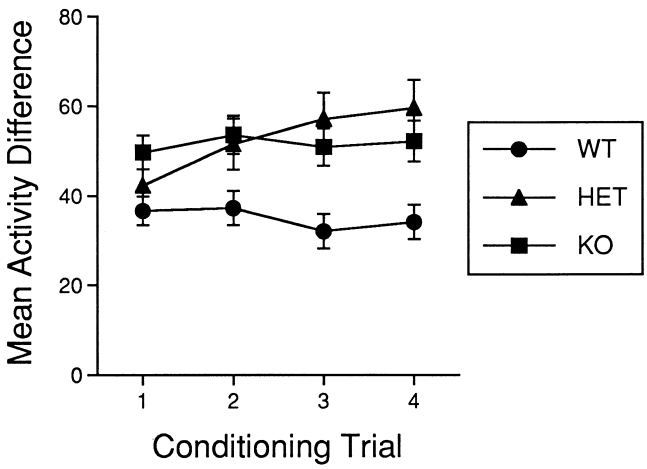

Fig. 2. Mean ± S.E.M. difference in activity counts/min between each CS+ and the corresponding CS − trials. These difference scores were derived from data shown in Fig. 1.

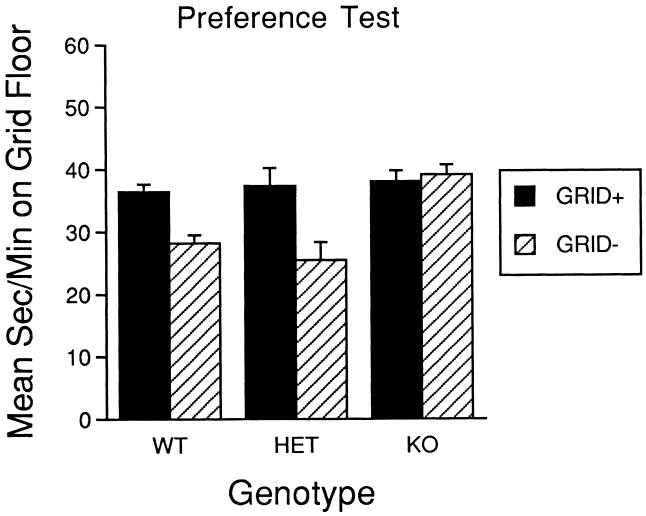

Fig. 3. Mean ± S.E.M. s/min spent on the grid floor during the 30-min test session (collapsed across Experiments 1 and 2). Mice in the GRID+ conditioning subgroups had previously received ethanol (2 g/kg) immediately before 5-min exposure to the grid floor on CS+ trials; saline was paired with the hole floor on CS − trials. These contingencies were reversed for mice in the GRID − conditioning subgroups. The numbers of mice in each conditioning subgroup were WT = 62, 68; HET = 19, 19; and KO = 48, 45.

As expected, saline-treated control groups spent about half of the test session on each floor. Mean ± S.E.M. s/min on the grid floor was 31.2 ± 1.7 and 30.1 ± 4.2 for the WT and KO control groups, respectively. There was no significant difference between genotypes [$F(1,21) < 1$]. Because these data were collected only in Experiment 1, which included only KO and WT mice, similar data were not collected in HET mice.

Activity levels during the preference test mirrored genotype differences observed on saline conditioning trials. Mean ± S.E.M. activity rates were 41.5 ± 1.1, 29.0 ± 1.7 and 21.9 ± 0.8 for WT, HET and KO mice, respectively. One-way ANOVA showed a significant genotype effect [$F(2,258) = 89.1$, $P < .0001$]; follow-up comparisons indicated that all pairwise differences were significant (Bonferroni-corrected P's < .005).

3. Discussion

These studies show that deletion of the dopamine D2 receptor gene interferes with ethanol-induced conditioned place preference in mice. In fact, under conditions that produced a reliable conditioned preference in WT mice, D2 receptor-deficient mice showed no evidence of place conditioning. HET mice performed similarly to WT mice, suggesting that one functional D2 receptor allele is suffi-

cient to support ethanol place conditioning. The general pattern of results, including the similarity of WT and HET mice, is quite consistent with previously reported findings of reduced intake and preference of ethanol by D2 KO mice in a home cage ethanol-drinking task [23]. Moreover, the direction of the place conditioning difference supports the hypothesis that reduced intake/preference by D2 KO mice reflects a decrease rather than an increase in ethanol reward. Taken together, these studies strongly support the conclusion that D2 receptors normally influence ethanol's rewarding effects in mice.

Despite use of a new genetic background (B6 × DBA/2 F2 hybrid), activity differences on saline (CS −) trials were generally consistent with basal open-field activity differences previously reported in mice carrying the mutation on a B6, 129 or B6 × 129 F2 hybrid background [1,20,23]. That is, the mutation produced a gene dose-related reduction in activity. However, the ethanol activity effect was quite different from those previously found in incipient congenic B6 mice lacking D2 receptors. In contrast to that earlier study, which showed ethanol-induced suppression of activity in WT and HET mice [23], the present study showed ethanol-induced activation in all three genotypes. Moreover, after correction for differences in basal activity, the present study revealed greater activation in KO and HET than in WT mice (Fig. 2). Because ethanol dose and concentration were identical in these studies, the difference in ethanol's activity effect is most likely due to differences in genetic background. More specifically, it seems likely that enhanced sensitivity to ethanol's activating effect in the present study reflects the influence of alleles from the DBA/2 strain, which is well-known to be more sensitive to ethanol-induced activation than the B6 strain (e.g., [6,13,24]). Nevertheless, the present findings are generally consistent with those of Phillips et al. [23] if one assumes that reduced depressant effects of ethanol in their KO mice reflected an enhanced sensitivity to ethanol's stimulant effects, as those authors proposed.

Although ethanol produced activation in all three genotypes, repeated exposure failed to produce sensitization to ethanol's locomotor stimulant effect (see Fig. 2). This outcome contrasts with the usual finding of locomotor sensitization in DBA/2 mice during ethanol place conditioning [3,8,9,12,14,25]. Thus, with respect to sensitization, it appears that the hybrid WT genotype is more similar to the B6 strain, which typically displays no locomotor sensitization [8]. The lack of sensitization in WT mice precludes conclusions about a possible role of D2 receptors in that effect.

Contrary to predictions of psychomotor stimulant theory [33], comparison of the pattern of genotype effects on ethanol-induced activation and conditioned place preference suggests a dissociation between these phenotypes. That is, a stronger stimulant response to ethanol was not necessarily predictive of stronger conditioned place preference. For example, although ethanol-stimulated activity was significantly lower in WT than in HET mice, both genotypes showed equivalent place preference. Moreover, despite showing similarly high levels of ethanol-induced stimulation, KO and HET mice differed in place preference. In general, this dissociation between ethanol's activating and rewarding effects is quite consistent with findings from previous ethanol place conditioning studies (e.g., [8,26]).

The present study also suggests a dissociation between test session activity levels and expression of conditioned preference. For example, even though HET mice showed significantly lower basal activity than WT mice, both genotypes expressed equivalent conditioned place preference. Moreover, given previous genetic correlational analyses showing that low levels of test activity are associated with strong conditioned place preference [8], the failure to obtain conditioned place preference in KO mice is not easily attributed to their low activity levels. Rather, as suggested earlier, that failure is better explained by reduction in ethanol's rewarding effects.

Previous quantitative trait locus (QTL) analyses of ethanol place conditioning in BXD recombinant inbred mouse strains (originally derived from B6 × DBA/2 F2 hybrids) have yielded a provisional QTL on chromosome 9 in the vicinity of the dopamine D2 receptor gene [8]. Although there has not yet been any verification that a functional polymorphism exists in this gene or that differences in its expression influence conditioned place preference, the present findings certainly encourage further consideration of that possibility.

Results of the present studies are not consistent with previous studies that failed to show an effect of acute pharmacological blockade of D2 receptors on either learning [25] or expression [12] of ethanol-conditioned place preference. The reasons for this disparity are unknown. Conclusions from those previous studies, however, must be tempered by the fact that only a single inbred genotype (DBA/2) was studied using only one antagonist (haloperidol) and a limited range of doses. Another possibility, of course, is that the present findings depended on a chronic (lifelong) loss of D2 receptor function that cannot be mimicked by acute pharmacological blockade.

One aspect of the place conditioning results in KO mice is somewhat puzzling. Specifically, although absence of a GRID+ vs. GRID − subgroup difference indicated a lack of conditioning in these mice [7], both subgroups showed an overall preference for the grid floor (64% time on grid). In contrast, saline-treated KO mice showed no floor preference (50% time on grid). This difference between saline- and ethanol-treated mice suggests that a history of repeated ethanol exposure alters unconditioned floor preferences in D2 receptor-deficient mice. Although the mechanisms underlying this preference shift are unknown, this finding underscores the importance of using a counterbalanced discrimination or unpaired drug design in studies of conditioned place preference [7]. In many studies, place conditioning produced by alleged rewarding drugs is indexed by

reference to the performance of saline-treated control subjects. In the present case, that strategy might have encouraged the erroneous conclusion that ethanol produced a conditioned place preference in KO mice. However, the discrimination design used here, which matches subgroups for overall exposure to ethanol, saline and both CSs, clearly shows there was no effect of the CS − ethanol contingency in KO mice.

An important issue not addressed by the present study is whether effects of this mutation are limited to learning tasks that involve ethanol. Previous studies with D2 receptor-deficient mice have suggested specificity in effects of this mutation. For example, KO mice (incipient congenic B6) do not differ from WT mice in % correct arm choice when tested in a food-reinforced "win-stay" T-maze task (Low, unpublished results). In another set of studies involving null mutants created in a different laboratory, KO mice (B6 × 129/Sv F2 hybrid) were deficient in morphine-induced place conditioning but not in place conditioning produced by food reward [21]. In a more recent series of studies involving the same incipient congenic B6 mutant strain used to breed our mice, KO mice developed a significant morphine-conditioned place preference, but only when they were drug naive prior to conditioning and not when they were opiate-dependent and withdrawn [17]. Although the reasons for the disparate effects of the mutation on morphine-induced place conditioning in drug-naive mice remain unknown, the overall pattern of findings suggests that D2 receptor deficiency does not simply impair all types of learning.

The present studies illustrate the potential value of using the place conditioning task to help interpret mutation-induced differences in ethanol drinking. It is important to note, however, that ethanol drinking and conditioned place preference are influenced by multiple genes and environmental variables. Thus, interpretation of strain differences across these tasks can sometimes be complicated. The present studies suggest that mutation of the D2 receptor gene influences conditioned place preference in the same way it affects ethanol drinking, i.e., by reducing ethanol's rewarding effects. However, one must be cautious in interpreting these data more generally as evidence of a genetic relationship between ethanol drinking and conditioned place preference. Indeed, recent studies in the BXD recombinant inbred strains have not found a significant genetic correlation between these phenotypes [22], although the observed positive trend between ethanol preference and conditioned place preference is certainly consistent with the present findings.

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