



## Dopamine D2R DNA transfer in dopamine D2 receptor-deficient mice: Effects on ethanol drinking

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### Abstract

Dopamine (DA) signals are transmitted via specific receptors including the D2 receptors (D2R). Previous studies have shown that D2R upregulation in the nucleus accumbens (NAc) attenuated alcohol consumption. We hypothesized that upregulation of D2R in the NAc would significantly influence alcohol drinking. We tested this hypothesis by determining the effect that D2R upregulation has on alcohol intake in genetically altered mice lacking D2Rs. After a steady baseline of drinking behavior was established for all mice, a null vector or a genetically modified adenoviral vector containing the rat D2R cDNA was infused into the NAc of wild-type (*Drd2*<sup>+/+</sup>), heterozygous (*Drd2*<sup>+/-</sup>), and receptor-deficient mice (*Drd2*<sup>-/-</sup>). Ethanol intake and preference were then determined using the two-bottle choice paradigm. Our results indicated that *Drd2*<sup>+/+</sup> mice treated with the D2R vector significantly attenuated (58 %) their ethanol intake as well as reduced preference. *Drd2*<sup>+/-</sup> and mutant mice showed a similar attenuation, although the change was not as marked (12 %) and did not last as long. In contrast, *Drd2*<sup>-/-</sup> mice treated with the D2R vector displayed a temporary but significant increase (46 %) in ethanol intake and preference (consumption). These results supported the notion that the D2R plays an important role in alcohol consumption in mice and suggest that a key threshold range of D2R levels is associated with elevated alcohol consumption. Significant deviations in D2R levels from this range could impact alcohol

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consumption, and could help to explain possible individual variations in alcohol response, metabolism, sensitivity and consumption.

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## Introduction

Alcoholism is one of the most widespread diseases of modern times. While significant advances have been made at understanding the mechanism(s) of alcohol abuse and addiction, much remains unanswered. Brain dopaminergic receptor systems are thought to be important for the rewarding effects of alcohol (Koob et al., 1998; Samson and Harris, 1992), and it has been proposed to be one of the neurotransmitters that modulate the predisposition to alcohol abuse (Di Chiara et al., 1996; George et al., 1995; Li, 2000). Previous research has shown that the reinforcing effects of alcohol involve among others the projections of DA cells to the NAc (Koob et al., 1987). In particular the mesolimbocortical DA pathway arising from the ventral tegmental area (VTA) and innervating the ventral striatum and the NAc, plays a role in incentive motivational processes (Horvitz, 2000).

Several studies have reported that the D2R is involved in transmitting the DA mediated reinforcing effects of alcohol (McBride et al., 1993a; Nowak et al., 2000; Stefanini et al., 1992). Clinical studies have implicated the role of D2Rs in alcoholism (Guardia et al., 2000; Lu et al., 2001; Matsumoto et al., 2001; Volkow et al., 1996a) and chronic alcohol consumption is associated with significant reductions in D2R concentrations (Guardia et al., 2000; Tajuddin and Druse, 1996; Volkow et al., 1996b, 2002). This has led to the hypothesis that low levels of D2R may predispose subjects to alcohol and drug use as a mean to compensate for the decrease in activation of reward circuits activated by these receptors (Blum et al., 1996; Volkow et al., 1999a,b, 2002). Several animal studies have supported these findings and have reported that D2R antagonists enhanced ethanol self-administration in selectively bred alcohol-preferring P rats (Dyr et al., 1993; Levy et al., 1991). In addition, it has been demonstrated that inbred ethanol preferring rats displayed lower D2R binding versus non-preferring rats (McBride et al., 1993a,b; Stefanini et al., 1992; Thanos et al., 2004). Recently it was demonstrated that D2Rs in the NAc participated in coding for a specific type of neural response to incentive contingencies and associative or spatial learning (Tran et al., 2002). Specifically, disruption of the D2R in the mesolimbocortical DA pathway changed neural responses to incentive stimuli in *Drd2*<sup>-/-</sup> mice. The strong bias to place-related neural activity in NAc neurons in the *Drd2*<sup>-/-</sup> mice suggested that neural substrates for spatial mapping of the environment were compromised in mice lacking the D2R (Tran et al., 2002).

DNA transfer (adenoviral) of the D2R into the NAc of adult Sprague Dawley rats, previously trained to self-administer alcohol, produced marked reductions in alcohol preference and consumption, which normalized after D2R gene expression returned to baseline levels (Thanos et al., 2001). Similarly, inbred ethanol preferring P rats treated with the D2R vector (in the NAc) significantly attenuated their alcohol preference (37 % decrease) and intake (48 % decrease), and these measures returned to pretreatment levels by day 20 (Thanos et al., 2004). In addition, chronic (7 weeks) ethanol exposure in P and NP rats

demonstrated a comparable D2R profile (Thanos et al., 2004) than was previously observed in naïve P and NP rats (McBride et al., 1993a).

Transgenic and gene knockout models including *Drd2*<sup>-/-</sup> mice have provided further insight into the genetically mediated differences in susceptibility to ethanol's effects, aiding our understanding of the genetic mechanism of alcohol abuse and alcoholism (Palmer et al., 2003). Specifically, *Drd2*<sup>-/-</sup> mice have been shown to have decreased preference and sensitivity to ethanol when compared with *Drd2*<sup>+/+</sup> (Phillips et al., 1998) but show a greater susceptibility to the development of locomotor sensitization with repeated ethanol treatment (Palmer et al., 2003). While these data provided powerful evidence of the involvement of the D2R in ethanol consumption, interpretation of the results requires further study, since *Drd2*<sup>-/-</sup> mice have a total absence of D2R throughout the brain; which may influence ethanol drinking in a different manner than localized D2R upregulation or downregulation. To start with, the absence of D2R will impede the animal's ability to learn an association between alcohol-induced increases in DA and the responses that are triggered by D2R activation. Also the potential role of altering local D2R concentrations on brain circuits involved with the rewarding responses to alcohol needs to be examined in these animals. Therefore, it is essential to study the effects of local D2R restitution on the sensitivity to alcohol in these *Drd2*<sup>-/-</sup> mice.

The purpose of this study was to determine the effects of manipulating D2R number in the NAc by DNA transfer techniques using a replication-deficient adenoviral vector containing cDNA coding for the D2R (AdCMV.D2R) on alcohol consumption in *Drd2*<sup>-/-</sup> mice compared to *Drd2*<sup>+/-</sup> and *Drd2*<sup>+/+</sup> mice. DNA transfer via adenoviral vector has been an effective strategy to introduce particular genes into tissue and has provided a high specificity targeting and delivery (Crystal, 1992; Suhr and Gage, 1993). The effectiveness of the present vector for intracerebral transfer of D2R as well as the expression of functional D2R effects has been previously well established (Ikari et al., 1995, 1999; Ingram et al., 1998; Ogawa et al., 2000; Thanos et al., 2001; Umegaki et al., 1997). Rats infected with this adenoviral vector produced significant increases in D2R expression with significant decreases in alcohol preference and intake (Thanos et al., 2001, 2004).

We hypothesized that upregulation of D2R in the NAc (by treatment with the D2R vector), would influence ethanol intake and preference depending on the animal's D2R status. More specifically we predicted that ethanol intake and preference would increase in the receptor-deficient mice receiving vector plus cDNA sequences and decrease in *Drd2*<sup>+/-</sup> and *Drd2*<sup>+/+</sup> mice.

## Materials and methods

All studies were conducted in accordance with the guidelines established by the National Institutes of Health in *The Guide for Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Brookhaven National Laboratory. Mice were individually housed in a room controlled for temperature and humidity as well as a 12-hour light/dark (lights off 0700h) cycle. Food, water and ethanol were provided ad libitum.

Adult male congenic (N10) on C57Bl/6J mice were used in this study [WT (*Drd2*<sup>+/+</sup>) HT (*Drd2*<sup>+/-</sup>) and KO (*Drd2*<sup>-/-</sup>)] and had a mean weight of  $35 \pm 4$  g. The present study consisted of two groups: 1) D2R vector treated and 2) Null vector treated. Each group consisted of 10 mice per strain (*Drd2*<sup>+/+</sup>, *Drd2*<sup>+/-</sup> and *Drd2*<sup>-/-</sup>).

### *Microsurgery and vector microinfusion*

Mice were anesthetized with an equal mixture of ketamine / xylazine (100 mg/kg / 10 mg/kg) and placed in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Mice were unilaterally microinfused with either the D2R or Null vector into the NAc according to stereotaxic coordinates in the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2001). The side of infusion into each mouse was selected randomly such that half received infusions into right NAc and half into left NAc.

Previous reports describe details of the vector construction (Ikari et al., 1995; Umegaki et al., 1997). Briefly, the recombinant Ad vectors were derived from Ad type 5 in which the majority of the E1 and a portion of the E3 regions had been deleted, and an expression cassette containing the rat D2R cDNA along with the cytomegalovirus (CMV) immediate early promoter and enhancer was inserted at the site of the E1 deletion. Two vectors were utilized: (1) D2R vector [(AdCMV.D2R) ( $10^{10}$  pfu/ml)], containing the rat D2R cDNA and (2) Null vector (AdCMV.Null), a similar construct, missing the D2R cDNA, as a control.

Microinfusion was carried out using an automated syringe pump (Razel, Stamford, CT) and a 26-gauge 1  $\mu$ l Hamilton microsyringe connected to a 28-gauge internal cannula. Each microinfusion administered 1  $\mu$ l of vector over 10 minutes so as to reduce the risk of procedure-induced lesions.

### *Behavior analysis: two bottle choice ethanol paradigm*

Ethanol self-administration was monitored using the two-bottle preference test, which is a model that replicates aspects of voluntary alcohol consumption in humans (McBride and Li, 1998). Animals were individually housed and had continuous access to both ethanol and distilled water from two 10 ml graduated glass drinking bottles. Mice were acclimated to the taste and effects of ethanol through progressively increasing the concentration of ethanol (0, 3, 6, and 10% v/v). After 2 weeks of habituation to the presence of 10% ethanol, baseline ethanol drinking behavior was recorded daily (1500h) for 2 weeks. Each day the position of the ethanol bottle was reversed to control for side preferences. During this time each animal's body weight was recorded. The amount of ethanol consumed divided by total fluid consumed  $\times$  100 was used as a measure of ethanol percent preference. In addition, daily ethanol consumption was measured in g/kg/day. After 2 weeks of baseline data, animals were treated with a microinfusion of either the D2R or the Null vector into the NAc, and then returned to the two-bottle choice ethanol procedure for 18 days.

## **Results**

### *D2R vector*

#### *Ethanol intake*

Baseline assessment of ethanol consumption in *Drd2*<sup>+/+</sup> mice revealed a relatively high level of ethanol consumption, with a baseline intake average of  $5.30 \pm 0.52$  g/kg/day (Fig. 1A). Treatment of *Drd2*<sup>+/+</sup> mice with D2R DNA transfer (D2R vector) resulted in a significant decrease (Fig. 1A) in ethanol drinking behavior (1-way repeated measures ANOVA;  $F = 7.23$ ;  $p < 0.001$ ). Subsequent

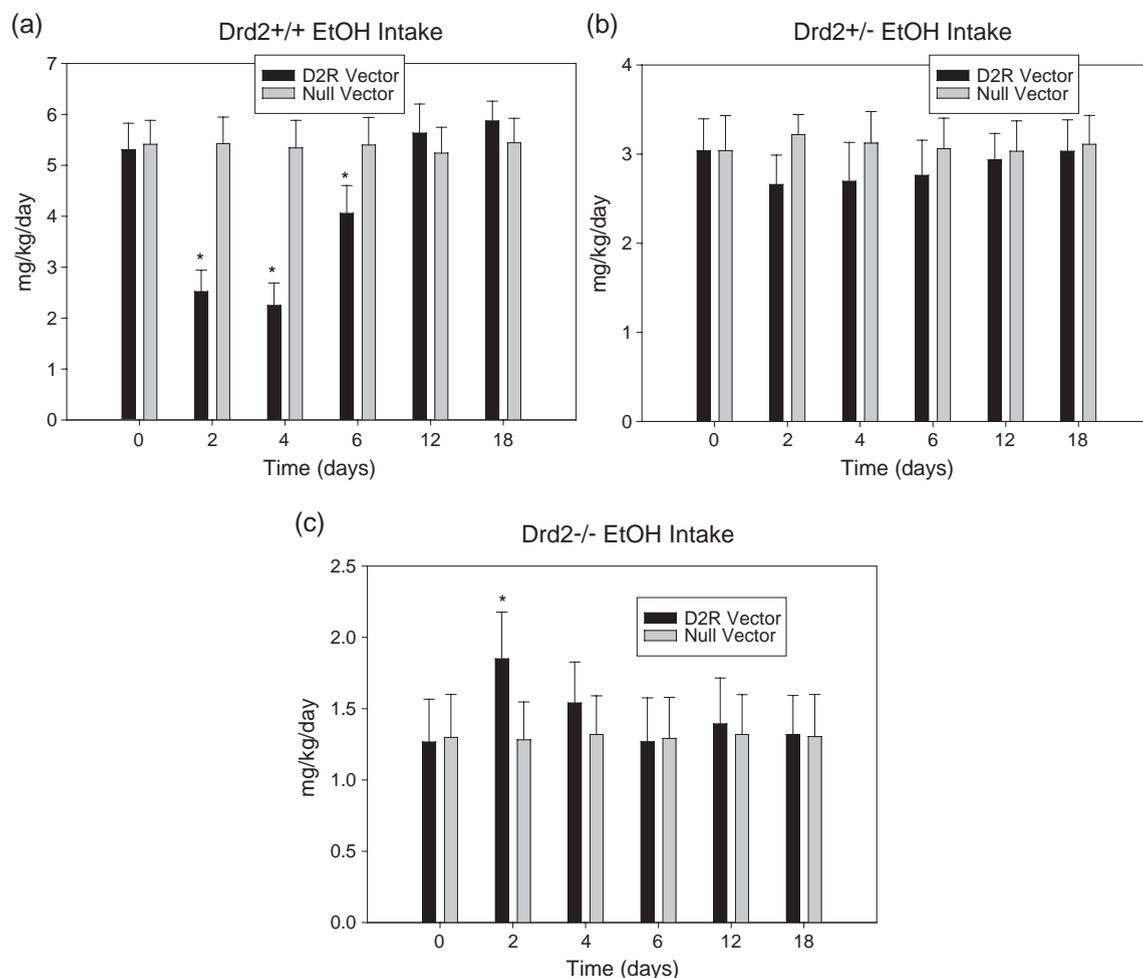


Fig. 1. Mean (+ SEM) ethanol intake (g/kg/day) over time (\*  $p < 0.05$ ) in: a) *Drd2*<sup>+/+</sup>; b) *Drd2*<sup>+/-</sup> and c) *Drd2*<sup>-/-</sup>.

analyses using paired t-tests revealed significant differences in ethanol intake between baseline (day 0) and post-D2R vector treatment, day 2 ( $t = 4.67$ ;  $p < 0.001$ ), day 4 ( $t = 4.42$ ;  $p < 0.002$ ) and day 6 ( $t = 1.25$ ;  $p < 0.04$ ). No significant differences were observed at 12 ( $t = 0.19$ ;  $p > 0.05$ ) or 18 days ( $t = 0.06$ ;  $p > 0.05$ ).

*Drd2*<sup>+/-</sup> mice displayed a baseline ethanol intake average of  $3.04 \pm 0.36$  g/kg/day (Fig. 1B). In these mice D2R DNA transfer did not produce a statistically significant change in ethanol consumption (1-way repeated measures ANOVA;  $F = 0.20$ ;  $p > 0.05$ ). Similarly, paired t-tests between baseline (day 0) ethanol intake and all post-D2R vector treatment days, did not reveal any statistically ( $p > 0.05$ ) significant differences (day 2,  $t = 1.13$ ; day 4,  $t = 0.69$ ; day 6,  $t = 0.57$ ; day 12,  $t = 0.20$ ; and day 18,  $t = 0.01$ ).

*Drd2*<sup>-/-</sup> mice revealed a baseline mean ethanol consumption of  $1.27 \pm 0.30$  g/kg/day (Fig. 1C). Paired t-test comparisons between baseline (day 0) and post-D2R vector treatment days, showed an initial significant increase in ethanol self-administration at day 2 ( $t = 2.76$ ;  $p < 0.02$ ). In contrast, paired t-

tests revealed no statistical difference ( $p > 0.05$ ) between baseline and day 4 ( $t = 0.27$ ), day 6 ( $t = 0.01$ ), day 12 ( $t = 0.36$ ) and day 18 ( $t = 0.15$ ).

### Ethanol preference

Percent ethanol preference was similarly assessed in all groups before and after treatment with D2R DNA transfer. In particular, *Drd2*<sup>+/+</sup> mice demonstrated a decline in mean % ethanol preference after treatment with the D2R vector (Fig. 2A). *Drd2*<sup>+/+</sup> mice exhibited a  $64.84 \pm 1.25$  % ethanol preference baseline average (day 0) and when compared to post D2R vector treatment a significant difference was observed (1-way repeated measure ANOVA;  $F = 11.08$ ;  $p < 0.001$ ). More specifically, multiple paired t-test comparisons revealed the following differences in % ethanol preference between baseline and day 2 ( $t = 2.739$ ,  $p < 0.02$ ); day 4 ( $t = 4.46$ ,  $p < 0.002$ ); day 6 ( $t = 5.38$ ,  $p < 0.001$ ); day 12 ( $t = 0.518$ ,  $p > 0.05$ ) and day 18 ( $t = 0.404$ ,  $p > 0.05$ ).

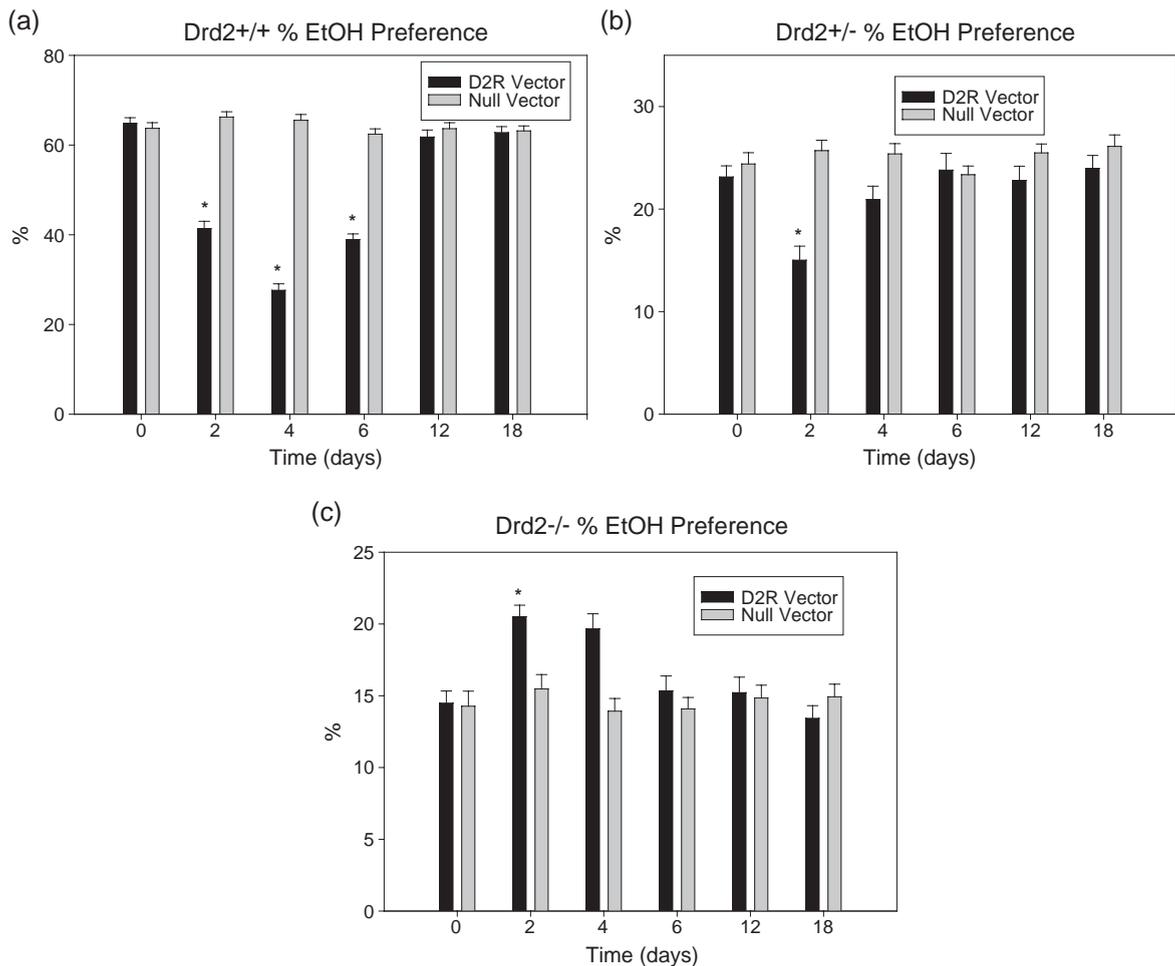


Fig. 2. Mean (+ SEM) percent ethanol preference over time (\*  $p < 0.05$ ) in: a) *Drd2*<sup>+/+</sup> ; b) *Drd2*<sup>+/-</sup> and c) *Drd2*<sup>-/-</sup>.

*Drd2*<sup>+/-</sup> ethanol % preference ( $23.13 \pm 1.08$ ) was not significantly influenced after D2R DNA transfer compared to baseline (1-way repeated measure ANOVA;  $F = 1.17$ ;  $p > 0.05$ ; Fig. 2B). However, subsequent multiple paired t-tests revealed the following differences in % ethanol preference between baseline and day 2 ( $t = 2.700$ ,  $p < 0.024$ ); day 4 ( $t = 0.522$ ,  $p > 0.05$ ); day 6 ( $t = 0.104$ ,  $p > 0.05$ ); day 12 ( $t = 0.070$ ,  $p > 0.05$ ) and day 18 ( $t = 0.364$ ,  $p > 0.05$ ).

Similarly, a 1-way repeated-measures ANOVA of the *Drd2*<sup>-/-</sup> ethanol % preference ( $14.48 \pm 0.84$ ) did not reveal a significant main effect of D2R DNA transfer ( $F = 1.887$ ;  $p > 0.05$ ; Fig. 2C). However, paired t-test comparisons revealed the following differences between baseline and day 2 ( $t = 2.261$ ,  $p < 0.05$ ); day 4 ( $t = 1.325$ ,  $p > 0.05$ ); day 6 ( $t = 0.311$ ,  $p > 0.05$ ); day 12 ( $t = 0.228$ ,  $p > 0.05$ ) and day 18 ( $t = 0.698$ ,  $p > 0.05$ ).

### Null vector

Quantitative analyses of ethanol intake and percent preference data in the Null vector treated mice did not reveal any significant changes (Figs. 1, 2). Using a 1-way ANOVA, ethanol intake post treatment with the Null vector did not significantly differ from baseline in the *Drd2*<sup>+/+</sup> ( $F = 0.008$ ;  $p > 0.05$ ), *Drd2*<sup>+/-</sup> ( $F = 0.044$ ;  $p > 0.05$ ) and *Drd2*<sup>-/-</sup> ( $F = 0.003$ ;  $p > 0.05$ ) mice. Similarly, a 1-way ANOVA was used to examine ethanol percent preference levels. Ethanol preference remained at similar to baseline levels, and did not differ statistically in the *Drd2*<sup>+/+</sup> ( $F = 0.091$ ;  $p > 0.05$ ), *Drd2*<sup>+/-</sup> ( $F = 0.099$ ;  $p > 0.05$ ) and *Drd2*<sup>-/-</sup> ( $F = 0.049$ ;  $p > 0.05$ ) mice.

## Discussion

Previous studies demonstrated that D2R deficient mice displayed altered levels of ethanol preference (Phillips et al., 1998), operant ethanol self-administration (Risinger et al., 2000), and ethanol conditioned place preference (Cunningham et al., 2000). In this study, we examined the effects of manipulating the levels of D2R gene expression in the NAc of *Drd2*<sup>-/-</sup> mice.

We observed that *Drd2*<sup>-/-</sup> mice displayed a lower baseline with respect to ethanol drinking compared to *Drd2*<sup>+/+</sup>, in agreement with previous studies (Phillips et al., 1998). Ethanol preference was 15% for *Drd2*<sup>-/-</sup> compared to 23% for *Drd2*<sup>+/-</sup> and 65% for *Drd2*<sup>+/+</sup>, and ethanol intake was 1.3 g/kg/day for *Drd2*<sup>-/-</sup> mice compared to 3 g/kg/day for *Drd2*<sup>+/-</sup> and 5.3 g/kg/day for *Drd2*<sup>+/+</sup> mice.

*Drd2*<sup>+/+</sup> mice treated with the D2R vector in the NAc displayed significant reductions in both ethanol preference (57%) and intake (58%), which was similar to the effects that the D2R vector treatment had on ethanol intake and preference in rats (Thanos et al., 2001, 2004). Maximum attenuation of both preference and intake was observed 4 days after treatment with the D2R vector and lasted approximately one week. D2R DNA transfer in *Drd2*<sup>+/-</sup> mice revealed an intermediate level of attenuation (35%) in ethanol preference. In contrast, D2R DNA transfer in the *Drd2*<sup>-/-</sup> mice increased ethanol intake (46%), and preference (42%). This effect was, however, short lasting with baseline drinking resuming by day 4 post treatment. We interpret the influence of genotype on the drinking behavior of C57Bl/6J following the manipulation of D2R number to imply that the presence of D2R is necessary for the expression of ethanol consumption, and that some threshold level of D2R expression in the NAc is required for the reinforcing effects of ethanol to occur. The differences in the baseline levels of alcohol consumption between the *Drd2*<sup>-/-</sup>, *Drd2*<sup>+/-</sup> and *Drd2*<sup>+/+</sup> mice and the

decreases in alcohol consumption with D2R over expression suggested an inverted u shape curve between the levels of D2R and alcohol consumption in these mice.

While examination of the role of D2R on conditioned association and learning was beyond the scope of the present study, it has been previously demonstrated that D2R in the NAc participates in the neuronal coding for specific types of responses to incentive contingencies and in spatial learning (Tran et al., 2002). More specifically, one of the two isoforms of the D2 receptor, termed D2L (long form) has been suggested to be critical in the acquisition (learning) and/or retention (memory) of context-stimulus associations (Smith et al., 2002) critical in understanding the neurobiological basis of alcoholism.

Future studies examining the effect(s) of chronic ethanol consumption on *Drd2*<sup>+/+</sup>, *Drd2*<sup>+/-</sup> and *Drd2*<sup>-/-</sup> mice will seek to assess whether the three genotypes differ with respect to their ability to learn to self administer alcohol, regardless of intake levels. Secondly, how if at all, is drinking behavior influenced long term (beyond 4 weeks post treatment); that is, if examined for a longer period of time, would *Drd2*<sup>+/-</sup> mice display an escalation in alcohol consumption? If our hypothesis remains intact D2R should be required for a conditioned association between drinking alcohol and its reinforcing effects to be formed, and that low D2R levels will inhibit learning this association, as well; total lack of D2R should produce even more pronounced deficit with respect to association learning. In addition, we hypothesize that once association learning has been established, reduced D2R levels should render the animal more vulnerable to more elevated levels of ethanol drinking. In the present study, *Drd2*<sup>+/-</sup> mice drank less than the *Drd2*<sup>+/+</sup> mice; which does not support our hypothesis; it is possible that conditions in the current study were not optimal to observe compulsive administration.

In summary, these results provide further insight into the complex role that D2R levels play in alcohol intake. Furthermore, these data help us better understand previous findings using D2R mice and differences in alcohol consumption, and support the notion of a critical threshold (Bmax) of D2R. It is possible that significant deviation from this optimum level, could be reflected behaviorally either as an attenuation or potentiation of alcohol drinking. In addition, these results may help us better understand the mechanism(s) of alcohol abuse and may well assist in the development of specific molecular based treatments of alcoholism. While the use of vector delivered sequences in alcohol research (as performed in this study) are very promising, several limitations remain (Israel et al., 2002). Future clinical studies involving the treatment of alcoholism using DNA transfer may provide an opportunity for utilizing less invasive, more specific, and safer approaches (through the use of nonviral or gutless vectors) (Israel et al., 2002). Also, as we learn more about D2Rs, and their involvement, in the mechanism of alcohol preference and addiction we will begin to better understand the relationship between the actual amount of D2R levels and ethanol drinking behavior, it may be possible to develop more effective interventions.

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