Neuropsychopharmacology (2015) 40, 1609–1618

www.neuropsychopharmacology.org

Upregulation of Dopamine D2 Receptors in the Nucleus Accumbens Indirect Pathway Increases Locomotion but Does Not Reduce Alcohol Consumption

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Brain imaging studies performed in humans have associated low striatal dopamine release and D2R binding with alcohol dependence. Conversely, high striatal D2R binding has been observed in unaffected members of alcoholic families suggesting that high D2R function may protect against alcohol dependence. A possible protective role of increased D2R levels in the striatum is further supported by preclinical studies in non-human primates and rodents. Here, we determined whether there is a causal relationship between D2R levels and alcohol intake. To this end, we upregulated D2R expression levels in the nucleus accumbens of the adult mouse, but selectively restricted the upregulation to the indirect striatal output pathway, which endogenously expresses D2Rs. After overexpression was established, mice were tested in two models of free-choice alcohol drinking: the continuous and intermittent access two-bottle choice models. As anticipated, we found that D2R upregulation leads to hyperactivity in the open field. Contrary to our expectation, D2R upregulation did not reduce alcohol intake during continuous or intermittent access or when alcohol drinking was tested in the context of aversive outcomes. These data argue against a protective role of accumbal indirect pathway D2Rs in alcohol consumption but emphasize their importance in promoting locomotor activity.
Neuropsychopharmacology (2015) **40,** 1609–1618; doi:10.1038/npp.2015.11; published online 4 February 2015

INTRODUCTION

There is considerable evidence implicating dopamine D2 receptor (D2R) function in the striatum in substance use disorders. Brain imaging studies performed in humans using positron emission tomography have consistently shown decreases in striatal dopamine release and D2R binding in alcohol dependence (Hietala *et al*, 1994; Volkow *et al*, 1996; Heinz *et al*, 2004; Martinez *et al*, 2005). The presumed decrease in D2R signaling that would be expected from reduced receptor levels may have important clinical implications. In alcoholics, low D2R binding is predictive of increased craving for alcohol, which correlates with a greater propensity to relapse (Heinz *et al*, 2005). In contrast, the high striatal D2R binding found in unaffected members of alcoholic families suggests that high D2R function may be protective against alcohol dependence (Volkow *et al*, 2006).

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Received 21 August 2014; revised 11 November 2014; accepted 23 November 2014; accepted article preview online 12 January 2015

On the basis of human studies, it has been difficult to determine whether decreased signaling at the striatal D2R reflects vulnerability to developing an addiction that was present before drug use, or whether it is a consequence of chronic drug intake. Support for both of these mechanisms can be found in animal models. Lower D2R availability measured by positron emission tomography in drug-naïve non-human primates predicts higher cocaine self-administration, but chronic self-administration also further lowers D2R availability (Morgan *et al*, 2002; Nader *et al*, 2006). Lower D2R availability in the ventral striatum is also associated with increased cocaine self-administration in rats that show impulsivity as a behavioral trait (Dalley *et al*, 2007). In the context of alcohol addiction, striatal D2R density is reduced in rat and mouse strains genetically selected for high alcohol consumption and the reductions in D2R density are observed before any exposure to alcohol (Stefanini *et al*, 1992; McBride *et al*, 1993; Bice *et al*, 2008).

These studies suggest that striatal D2R levels in drug-naïve animals can predict drug use at a later time point. However, establishing causality is critical as it may inform new strategies for preventing or reversing excessive drug use. Thanos *et al* (2001, 2004, 2005) have investigated this question by transiently upregulating D2R expression levels in the nucleus accumbens (NAc) in rodents using

adenoviral-mediated gene transfer. They found that D2R upregulation in the NAc core transiently reduced established alcohol drinking and preference in both wild-type and alcohol-preferring rodents. Although these results have significant implications for understanding the role of D2Rs in alcoholism, some important issues remain to be addressed. First, in these studies, D2Rs were overexpressed after alcohol drinking was already established, thereby demonstrating that D2R overexpression has direct consequences on ongoing excessive alcohol intake. However, whether increasing D2R in drug-naïve animals also confers later protection against excessive alcohol consumption is currently unknown and needs to be addressed. Second, the viral vector used in these previous studies is only transiently expressed, making it impossible to determine whether the acute effect of D2R overexpression on alcohol consumption would be maintained. Third, the neuronal cell type in which D2R overexpression may confer protection is unclear because the viral vector that was used can infect any cell type including medium spiny projection neurons (MSNs) as well as interneurons.

Striatal MSNs are organized into the direct and indirect projection pathways. The direct pathway predominantly expresses D1Rs and projects monosynaptically to the basal ganglia output nuclei, the internal segment of the globus pallidus and the substantia nigra pars reticulata. In contrast, the indirect pathway predominantly expresses D2Rs and modulates globus pallidus/substantia nigra pars reticulata output through a polysynaptic circuit via the external segment of the GP (Gerfen and Surmeier, 2011). Both pathways are functionally opposing with regard to thalamocortical activation and are therefore often referred to as 'Go' and 'NoGo' pathway. Although this separation is less distinct in the NAc compared with the dorsal striatum (Bertran-Gonzalez *et al*, 2008), activity in the direct and indirect pathway of the NAc have opposing influences on behaviors. In the context of drug addiction, sensitization and conditioned place preference to psychostimulants are generally promoted by the direct pathway but inhibited by the indirect pathway (Durieux *et al*, 2009; Ferguson *et al*, 2010; Lobo *et al*, 2010; Bock *et al*, 2013).

Thus, based on the previous work by Thanos *et al* and the known circuitry of the striatal output pathways, we sought to address two key questions left unanswered by previous studies: (i) When induced prior to alcohol exposure, does overexpression of D2Rs confer protection against alcohol drinking? (ii) Is overexpression that is selectively localized to the neurons of the indirect pathway, which endogenously express D2Rs, sufficient to provide protection? To this end, we used an adeno-associated (AAV) viral vector in combination with the Cre/loxP recombination system to produce long-lasting, stable D2R overexpression in the indirect pathway and measured its effect on subsequent alcohol consumption in two models of free-choice alcohol drinking.

MATERIALS AND METHODS

Mice

All procedures were approved by the Institutional Animal Care and Use Committee at Columbia University. Adult male *D2*-Cre BAC transgenic mice (ER44 line; GENSAT) backcrossed >10 generations onto the C57BL/6J background were singly housed under 12-h light/dark cycles with *ad libitum* food access during behavioral studies.

AAV Generation and Delivery

The AAV2/1-hSyn-DIO-D2R(L)-IRES-mVenus virus was generated by sub-cloning the sequence of interest (Figure 1a) under the human synapsin I promoter into the pAAV-EF1a-DIO-hChR2 plasmid (Addgene) packaged by Vector BioLabs (Philadelphia, PA). The control AAV2/5-hSyn-DIO-EGFP was purchased from the UNC Vector Core (University of North Carolina, Chapel Hill, NC). Mice were bilaterally injected at 8–9 weeks of age with 0.44 μl of D2R or EGFP AAVs using stereotactic coordinates: (AP, $+1.7$ mm; ML, ± 1.2 mm; DV, -3.9 mm). All experiments were conducted 4 weeks post injection.

Immunohistochemistry and Radioligand Binding Assay

Mice were deeply anesthetized and transcardially perfused with ice-cold 4% paraformaldehyde in PBS (Sigma, St Louis, MO). Brains were harvested and postfixed overnight. Free-floating 30-μm coronal sections were obtained using a Leica VT2000 vibratome (Richmond, VA). After incubation in blocking solution (10% fetal bovine serum, 0.5% bovine serum albumin in 0.5% TBS-Triton X-100) for 1h at room temperature, sections were labeled overnight at 4 °C with primary antibodies against GFP/mVenus (1 : 1000; Abcam, Cambridge, MA); D2R (1 : 500; in-house); choline acetyltransferase (1 : 100; Millipore, Temecula, CA); Cre-recombinase (1 : 3000; in-house), DsRed (1 : 250; Clontech, Mountain View, CA). Sections were incubated with fluorescent secondary antibodies for 1 h at RT and mounted on slides and coverslipped with Vectashield (Vector, Burlingame, CA). Digital images were acquired using a Nikon Meta Inverted confocal microscope and processed with NIH Image J and Adobe Photoshop software. A [^{3}H] N-methylspiperone binding assay was used to assess D2R ligand binding capacity in NAc membrane preparations, as previously done (Trifilieff *et al*, 2013).

Locomotor Activity

D2-Cre mice injected with D2R- or EGFP-expressing AAVs were tested in open field boxes equipped with infrared photobeams to measure locomotor activity (Med Associates, St. Albans, VT). Data were acquired using Kinder Scientific Motor Monitor software (Poway, CA) and expressed as total distance traveled (cm) over 90 min.

Voluntary Two-Bottle Drinking Procedures

Four weeks after AAV injection and 3 days after habituation to drinking water from two 50-ml polycarbonate tubes, single-housed mice ($n = 8$–9 per group) were given access to two bottles outfitted with ball point stainless steel tubes (Ancare, Bellmore, NY), containing either 3% ethanol in tap water (v/v) or tap water for 2 days. Each day, the bottle position was reversed to prevent location bias. For continuous access (CA), alcohol concentration was

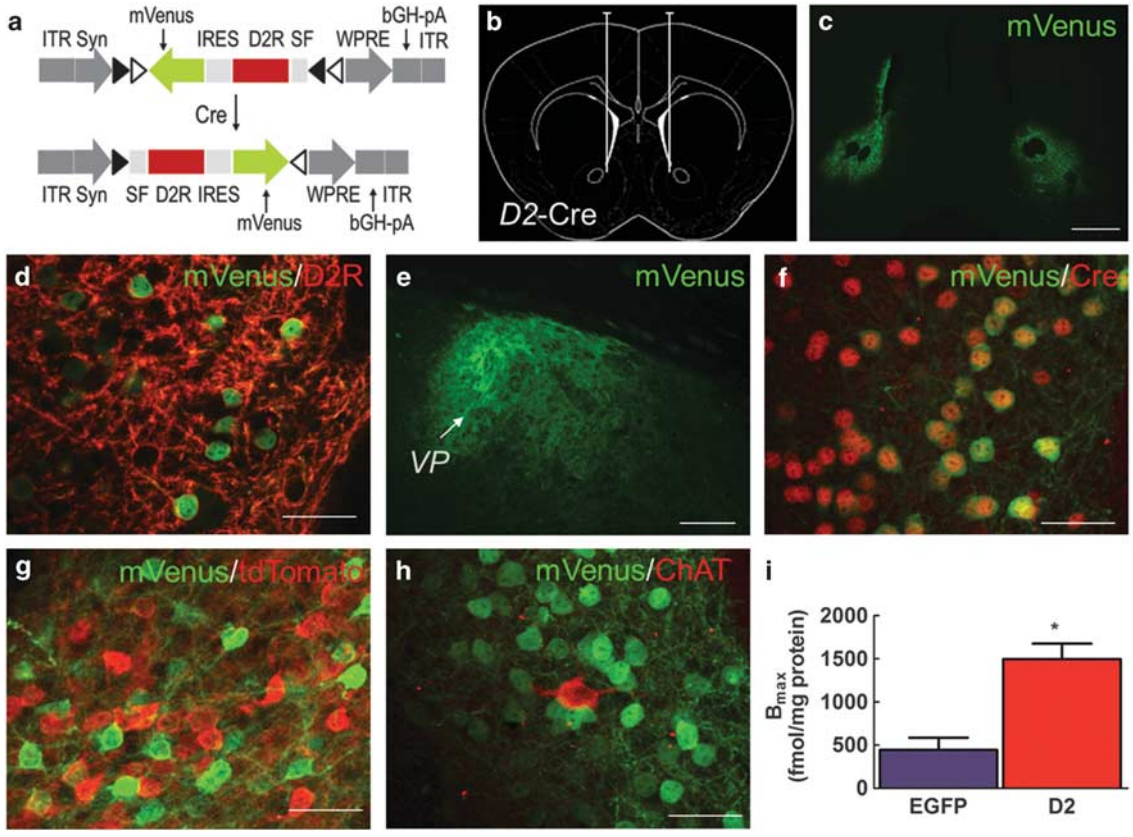

Figure 1 (a) AAV2/1 vector encoding a double-floxed inverted open reading frame (DIO) expressing D2R and the YFP variant mVenus under control of the synapsin promoter. ITR, inverted terminal repeat; IRES, internal ribosome entry site; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; bGH-pA, bovine growth hormone poly-A tail. (b) Schematic representation depicting bilateral injection site of AAV into NAc of D2-Cre mice. (c) mVenus expression in NAc 4 weeks post injection is primarily restricted to the core region. Scale = 600 μm. (d) Co-localization of upregulated D2R (red) with mVenus (green) was observed in membrane-like regions and processes. Scale = 50 μm. (e) Higher magnification image showing mVenus-labeled fibers in the ventral pallidum (VP), projection target of NAc indirect pathway. Scale = 150 μm. (f) Double-labeling of Cre and mVenus shows that mVenus expression is restricted to Cre-positive cells in *D2*-Cre mice. Scale = 50 μm. (g) Rare co-localization of tdTomato (D1-MSNs) and Venus (D2-MSNs overexpressing D2Rs) in the NAc of *D2*-Cre x *D1*-tdTomato mice 4 weeks after the AAV-D2R injection. (h) Choline acetyltransferase (ChAT), a marker for cholinergic interneurons rarely colocalizes with GFP-positive cells, in agreement with previous work in *D2*-Cre mice (Kravitz *et al*, 2010). Scale = 50 μm. (i) Ligand binding analysis with the D2 receptor antagonist [^{3}H]N-methyl-spiperone in NAc membranes shows significant upregulation in $D2R_{NAcInd}$ mice.

increased every 2 days in 3% increments until 30% for a total of 3 weeks. Mice and bottles were weighed daily to determine ethanol consumption and preference values over 24 h. Drip values were also obtained daily from bottles in an empty control cage and subtracted from the drinking values. Mice were also tested for their preference for either sucrose, saccharin or quinine hemisulfate (Sigma, St Louis, MO) *vs* water in a CA 2-bottle choice procedure. Saccharin and quinine experiments were done 8 and 10 weeks after AAV injection and lasted for 4 and 8 days, respectively. Sucrose experiments were run 4 weeks post injection and lasted 16 days. For the intermittent access (IA) two-bottle procedure, a different cohort of mice ($n = 7$–9 per group) was given every-other-day access to both 15% ethanol (v/v) and water as described (Melendez, 2011), 8 weeks post injection. A total of 10 IA sessions were conducted over 6 weeks. Each session consisted of two non-consecutive days of alcohol exposure; each day with the bottle locations reversed. Two water bottles were presented on intervening days. Quinine was introduced in the 15% alcohol solution immediately after the 10 IA sessions and its concentration increased with each subsequent session over 4 weeks.

Data Analysis

Data are expressed as mean ± SEM. Students' *t*-tests were used to compare between two groups. Multiple comparisons were evaluated by one-way or two-way repeated measures ANOVA, using GraphPad Prism software. Statistical significance was considered for $p < 0.05$.

RESULTS

Viral D2R Upregulation is Selective for D2R-Containing MSNs of the Adult NAc

We generated a Cre recombinase (Cre)-dependent AAV, which encodes a double-inverted open reading frame (DIO) containing the *Drd2* gene (long isoform), an internal ribosome entry site (IRES), and the YFP variant mVenus under the control of a synapsin promoter (Figure 1a). In order to selectively upregulate D2Rs in indirect pathway D2R-expressing MSNs (D2-MSNs), we bilaterally injected this vector in the NAc of adult *D2*-Cre mice (Figure 1b). After 4 weeks, immunohistochemical labeling of mVenus

revealed Cre-dependent viral expression in the NAc, predominantly in the core sub-region (Figure 1c). Expression was stable for at least 6 month after injection (Supplementary Figure S1). D2Rs and mVenus were co-expressed within cells with D2R expression enriched in neuropil (Figure 1d). Dense labeling of axonal terminals was observed in the projection target region of NAc D2-MSNs, the ventral pallidum (Figure 1e). Selective targeting of viral expression to D2-MSNs was confirmed by Cre co-labeling (Figure 1f). A small percentage (4.7%) of mVenus-positive cells co-expressed tdTomato in *D2*-Cre x *D1*-tdTomato mice (Figure 1g). This degree of co-expression is expected from published reports from our group and others which estimate that ~3–6% of NAc core neurons co-express D1Rs and D2Rs (Bertran-Gonzalez *et al*, 2008; Frederick et al, 2015). Striatal cholinergic interneurons are known to express D2Rs, so we examined whether cells expressing the cholinergic marker choline acetyltransferase were transduced by our vector. In agreement with previous work using this D2-Cre line (Kravitz *et al*, 2010), we found very few choline acetyltransferase-positive cells within the virus-transduced regions (Figure 1h). Only 5.6% of choline acetyltransferase-positive cells co-expressed mVenus or Cre (7.7%). Further, we quantified D2R levels in NAc membranes using a [^{3}H] N-methyl-spiperone binding assay (Figure 1i). Viral D2R upregulation in NAc led to ~threefold increase in binding capacity ($B_{max} = 1498.0$ fmol/mg protein) compared with EGFP-expressing NAc ($B_{max} = 443.4$; $t = 4.7$, $p = 0.01$, $n = 6$ mice (3 samples) per group).

D2R Upregulation in NAc D2-MSNs Leads to Hyperlocomotion

To determine whether indirect pathway-selective D2R upregulation (D2R-OE$_{NAcInd}$) leads to changes in NAc function *in vivo*, we first examined whether our manipulation would alter the balance of striatal control over locomotor activity. In the open field, D2R-OE$_{NAcInd}$ mice exhibited a near twofold increase ($t = 8.4$, $p < 0.0001$; $n = 23$–24 per group) in total distance traveled across the 90 min examined compared with control *D2*-Cre mice injected with a Cre-dependent AAV expressing EGFP (EGFP$_{NAcInd}$; Figure 2a and b). The D2R-OE$_{NAcInd}$ mice also displayed increased distance traveled in the center of the box and rearing behavior (Figure 2c–e; $t = 3.0$, $p < 0.005$ and $t = 2.6$, $p < 0.05$, respectively). These results show that increased D2R levels in the NAc indirect pathway lead to hyperlocomotion.

D2R Overexpression Increases Consumption of Fluids, Including Alcohol, in a CA Model of Voluntary Drinking

We tested the effect of D2R overexpression in the NAc on alcohol-related behavior using the well-documented two-bottle choice model of voluntary alcohol drinking (Richter and Campbell, 1940), in which mice drink freely from two bottles (alcohol *vs* water). Specifically, we examined ethanol intake and preference following CA to a range of increasing ethanol concentrations (3–30%) over the course of 3 weeks. Although intoxicating blood ethanol concentrations are rarely achieved with the CA model (Crabbe *et al*, 2011), this procedure has been valuable for characterizing basic drinking patterns across different mouse strains (Blednov *et al*, 2010).

With CA to increasing ethanol concentrations, both EGFP$_{NAcInd}$ and D2R-OE$_{NAcInd}$ mice progressively increased their daily alcohol intake, yet D2R-OE$_{NAcInd}$ mice consumed significantly more alcohol (Figure 3a and Supplementary Figure S2; $F_{(1,15)} = 14.3$, $p < 0.005$, two-way RM ANOVA, $n = 8$–9 mice per group). Despite their increased alcohol intake, preference for alcohol over water was not altered in D2R-OE$_{NAcInd}$ mice relative to controls (Figure 3b). In agreement with previous work in the C57Bl6 strain (Blednov *et al*, 2010), both groups showed peak alcohol preference between 9 and 12% ethanol and subsequently adjusted their preference at the higher ethanol doses. D2R-OE$_{NACInd}$ mice also exhibited a significant increase in total fluid consumption across both bottles ($F_{(1,15)} = 31.8$, $p < 0.0001$) and a small decrease in body weight compared with controls ($F_{(1,15)} = 3.93$, $p = 0.066$; Figure 3c and d). The increase in total fluid intake was evident even when two water bottles were presented prior to the introduction of ethanol (Figure 3c), demonstrating that the increased drinking behavior was not specific to alcohol.

To further examine this question, we tested drinking behavior in mice following pairings of sucrose, saccharin, or quinine *vs* water. Compared with controls, sucrose consumption was significantly higher in the D2R-OE$_{NACInd}$ group (Figure 3e, $F_{(1,15)} = 12.05$, $p < 0.005$). Saccharin and quinine intake were similarly increased, although not significantly (Figure 3f and g; saccharin: $F_{(1,15)} = 0.99$, $p = 0.336$; quinine: $F_{(1,15)} = 2.56$, $p = 0.13$). Preference tests for all three substances at increasing doses—as with alcohol—showed no significant differences between groups (Figure 3h–j). Together, the drinking data from the CA procedure suggest that D2R overexpression in D2-MSNs leads to an increase in fluid consumption that is not specific to alcohol. In addition, D2R overexpression does not alter gustatory preferences for sweet or bitter tastes (Figure 3h–j) known to strongly influence alcohol consumption (Blednov *et al*, 2008).

D2R Overexpression does not Modulate the Escalation of Alcohol Consumption in an IA Two-Bottle Choice Procedure

The CA two-bottle choice is limited in its ability to model important aspects of alcohol dependence, including escalation of drinking to intoxicating levels following periods of alcohol deprivation (Hwa *et al*, 2011). Therefore, we used an IA two-bottle procedure in which mice are given access to a fixed alcohol dose on alternating days over several weeks. The alternation between alcohol exposure and deprivation has been shown to lead to a rapid escalation in alcohol consumption in mice (Hwa *et al*, 2011; Melendez, 2011). In fact, the IA model is associated with withdrawal-like effects and leads to intoxicating blood alcohol levels, consistent with a dependent phenotype (Hwa *et al*, 2011).

We tested whether D2R upregulation in D2-MSNs would alter alcohol drinking of 15% alcohol when presented every other day for 6 weeks. Confirming the effectiveness of the IA procedure in leading to escalation of alcohol drinking, we found that both groups significantly increased their

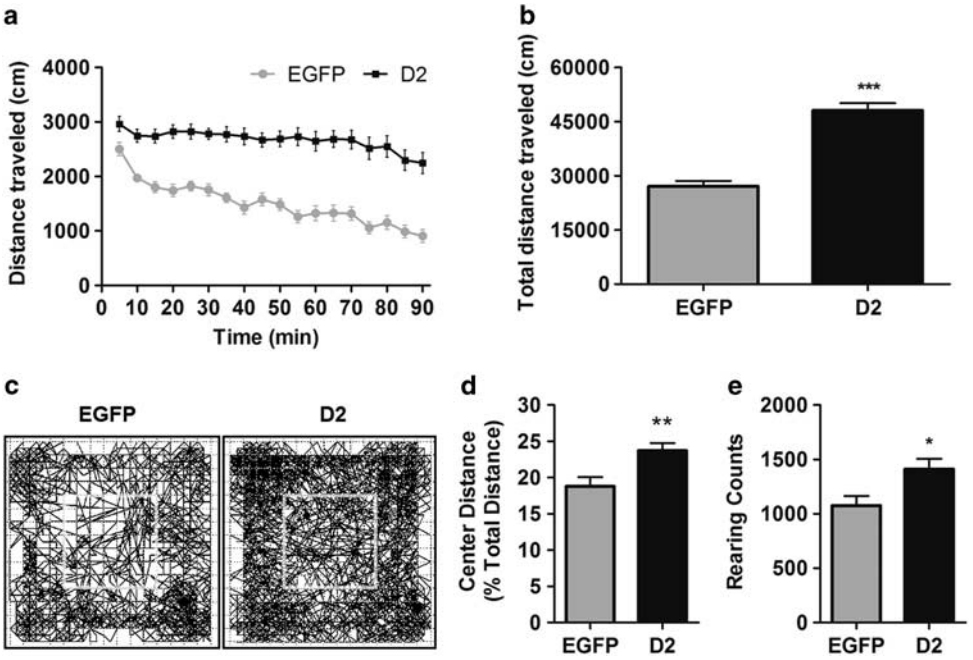

Figure 2 (a) Distance traveled in 5-min bins over a 90-min period by mice expressing EGFP or D2Rs in indirect pathway NAc MSNs. (b) Mean total distance traveled over the 90-min test period. (c) Representative horizontal activity tracks for the initial 45 min. Inner box represents the center zone. (d) Center distance, calculated as a percentage of the total distance, was significantly increased. (e) D2R-OE_{NAcInd} mice also displayed increased rearing behavior. *Statistically significant.

alcohol intake throughout the 10 IA sessions, with control and D2R-OE_{NAcInd} mice achieving mean doses of 17.3 mg/kg (51.9% increase) and 18.9 mg/kg (41.5% increase), respectively (Figure 4a and b; EGFP: $t = 11.4$, $p < 0.0001$; D2: $t = 3.55$, $p < 0.01$, $n = 7$–9 per group). However, we found no overall difference between groups. D2R-OE_{NAcInd} mice showed a small, but significant, reduction in preference for alcohol (Figure 4c, $F_{(1,14)} = 6.3$, $p < 0.05$, two-way RM ANOVA, $n = 7$–9 per group). This effect on preference was due to increased water consumption because D2R-OE_{NAcInd} exhibited increased total fluid consumption ($F_{(1,14)} = 20.8$, $p < 0.001$) but similar alcohol intake compared with controls (Figure 4d and Supplementary Figure S2). These data suggest that D2R upregulation in the NAc does not alter the escalation of alcohol consumption induced by this procedure.

D2R Overexpression does not Modulate the De-Escalation of Alcohol Consumption when Drinking is Paired with a Negative Outcome

Chronic alcohol intake in mice has been shown to lead to increased tolerance to the aversive effects of quinine when mixed with alcohol (Lesscher *et al*, 2010). Continued drinking of quinine-adulterated alcohol, despite its aversive properties, is thought to model the compulsive intake characteristic of alcohol dependence (Lesscher *et al*, 2010). We therefore determined whether excess D2Rs may affect how much alcohol the mice will continue to drink in the presence of increasing concentrations of quinine.

To this end, both groups of mice, which had been previously subjected to the IA procedure, were given access to 15% alcohol adulterated with concentrations of quinine ranging from 0.03 to 0.6 mM. Figure 5a and b show that drinking in both groups was largely resistant to quinine-containing alcohol at quinine concentrations that we and others had previously demonstrated to be aversive in the quinine preference test (Figure 3j; (Blednov *et al*, 2010), supporting the validity of the IA procedure in modeling compulsive-like aspects of alcohol dependence. A similar gradual reduction in alcohol consumption was observed in both groups with increasing quinine (Figure 5a). Preference for quinine-containing alcohol was reduced in the D2R-OE_{NAcInd} mice compared with controls (Figure 5B, $F_{(1,14)} = 8.46$, $p < 0.05$). As in Figure 4c, this genotype effect is likely due to the increased water intake, and not due to a reduction in alcohol intake (Figure 5c and Supplementary Figure S2). While EtOH preference is reduced in D2R-OE_{NAcInd} mice, increasing quinine doses led to similar relative changes in preference in both groups (Supplementary Figure S3). Together, the intake and preference data suggest that D2R upregulation does not modulate de-escalation of drinking behavior in the face of an aversive outcome.

DISCUSSION

We used Cre-mediated somatic recombination with AAV gene transfer to selectively overexpress D2Rs in the indirect pathway MSNs of the NAc. We found that this manipulation led to a twofold increase in locomotor activity in the open field, but contrary to our original hypothesis, it did not lower alcohol intake in two different models of voluntary alcohol drinking.

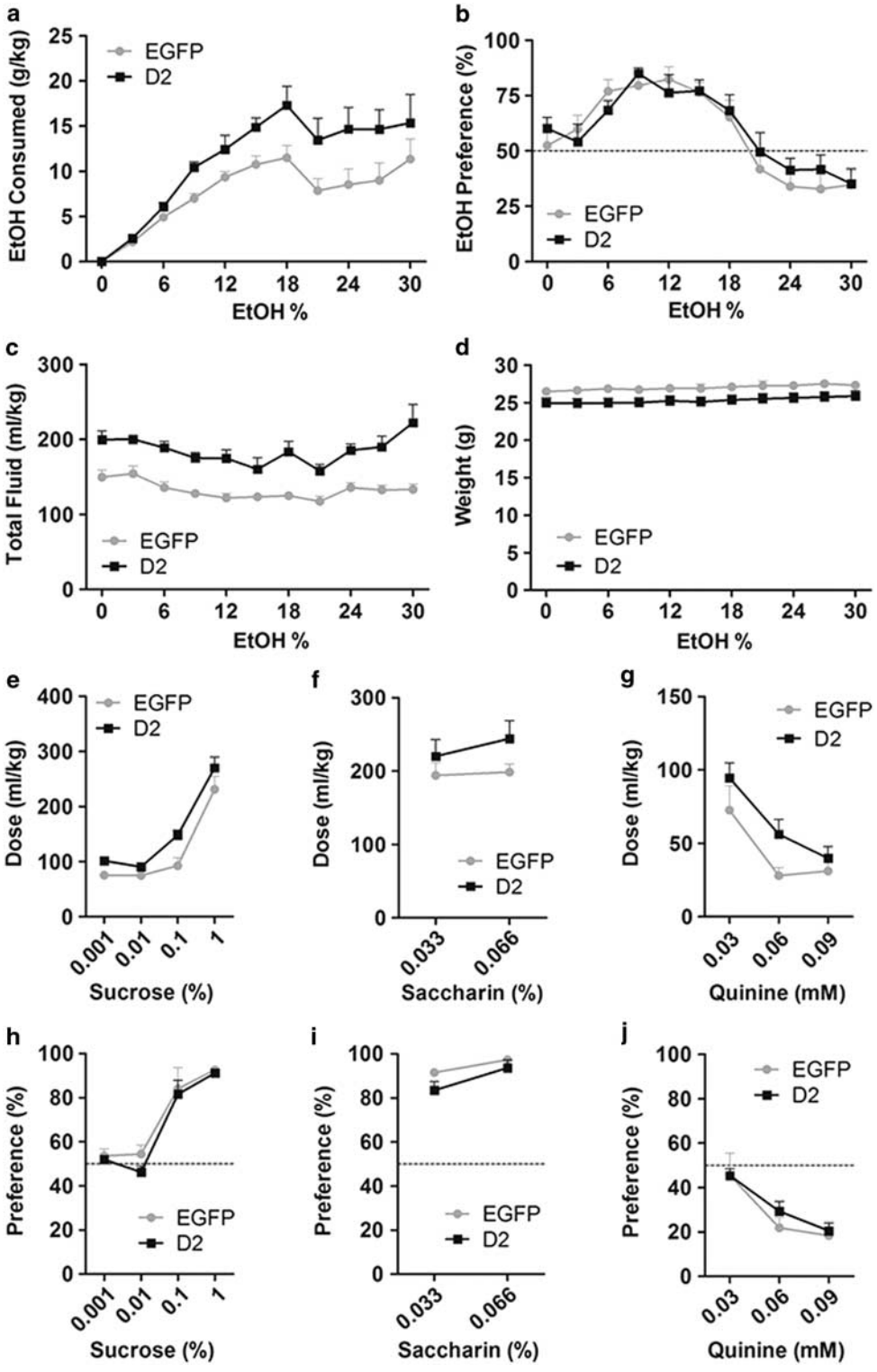

Figure 3 (a) Ethanol consumption is increased as a function of ethanol concentration (v/v) in a continuous access, two-bottle choice procedure in both groups. Ethanol consumption is significantly higher in D2R-OE_{NAcInd} mice compared with controls expressing EGFP. Data for each ethanol concentration were obtained as an average of 2 days of drinking ($p < 0.005$). (b) Both groups similarly adjust their preference of ethanol over water across the different ethanol concentrations. (c) Total fluid consumption (ethanol + water), while stable in both groups, is increased in D2R-OE_{NAcInd} mice ($p < 0.00001$). (d) D2R overexpression results in lower body weights ($p = 0.066$). (e–g) Dose-dependent consumption of sucrose, saccharin, and quinine alone when tested against water. Sucrose consumption is significantly increased in D2R-OE_{NAcInd} mice ($p < 0.005$). (h–j) Preference for sucrose, saccharin, or quinine over water is not altered by D2R upregulation.

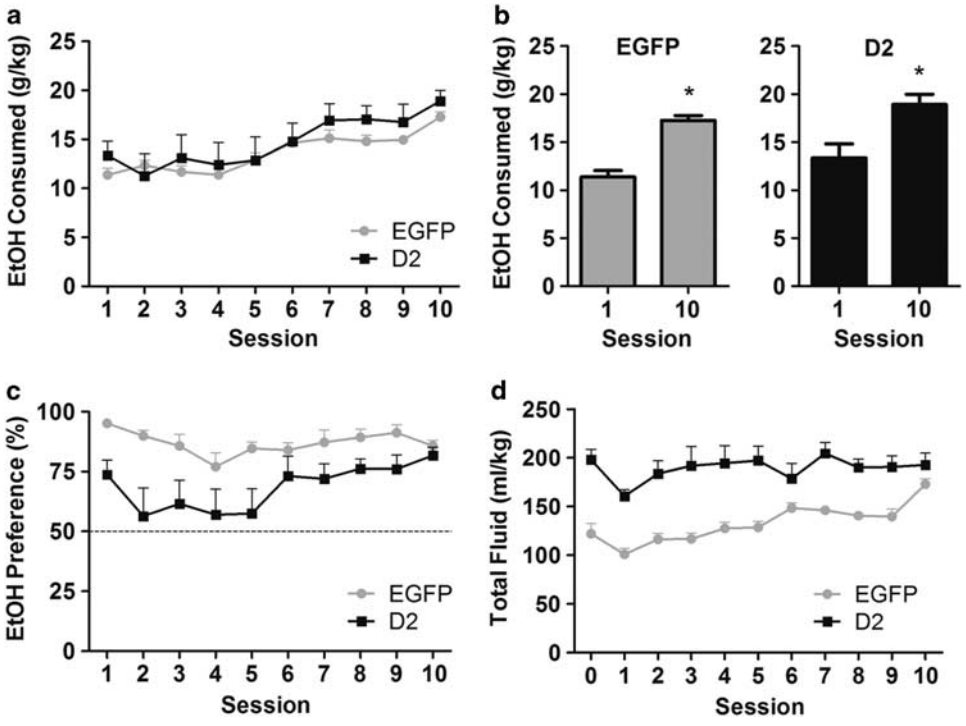

Figure 4 (a) Intermittent access (IA) to 15% ethanol *vs* water leads to a progressive escalation of ethanol intake over 10 sessions (20 intermittent days of ethanol exposure). Each session reflects the average consumption over 2 days of alcohol drinking in which alcohol bottle position was alternated to prevent side bias. D2R upregulation does not alter escalation of ethanol consumption. (b) The IA procedure effectively leads to a significant increase in ethanol consumption in both groups between the first and last session. (c) Although both groups prefer 15% ethanol over water, a reduction in preference was observed in the D2R-OE_{NAcInd} group compared with controls ($p<0.05$). (d) D2R upregulation also led to increased total fluid consumption ($p<0.001$).

D2R Upregulation in the Indirect Pathway Leads to Increased Locomotor Activity in the Open Field

The direct and indirect striatal output pathways are thought to create a dynamic balance, exerting opposing but concerted actions on the control of movement, cognition and motivational processes (Gerfen and Surmeier, 2011; Cui *et al*, 2013). In line with this model, artificial stimulation of the indirect pathway using optogenetic tools inhibits locomotor activity in the open field (Kravitz *et al*, 2010; Cazorla *et al*, 2014). Conversely, selective lesion of the indirect pathway increases locomotion (Durieux *et al*, 2011). In the classical model of Parkinson's disease, D2Rs are thought to inhibit the indirect pathway activity and thus, D2R hyperfunction should disinhibit locomotor initiation and function (Gerfen and Surmeier, 2011). Our observations in the open field are consistent with this model. When D2Rs are upregulated in the indirect pathway, locomotor activity is enhanced. Our results differ from the described optogenetic and selective lesion studies in that we targeted the NAc core instead of the dorsomedial striatum (Kravitz *et al*, 2010; Durieux *et al*, 2011; Cazorla *et al*, 2014). However, numerous pharmacological studies using local infusions have demonstrated a role for dopamine in the NAc core in regulating open field activity (Pulvirenti *et al*, 1994; Swanson *et al*, 1997; Canales and Iversen, 2000; Baldo *et al*, 2002). Our results are consistent with these findings and suggest that the D2R in the accumbal indirect pathway is a critical regulator of locomotor activity and indicate that the virally expressed D2Rs are functional.

D2R Upregulation in the Indirect Pathway does not Decrease Alcohol Drinking

On the basis of the human imaging studies (Hietala *et al*, 1994; Volkow *et al*, 1996; Heinz *et al*, 2004; Martinez *et al*, 2005; Volkow *et al*, 2006), the psychostimulant studies in monkeys and rats (Morgan *et al*, 2002; Nader *et al*, 2006; Dalley *et al*, 2007), and the viral overexpression studies in rats and mice using adenoviruses (Thanos *et al*, 2001, 2004, 2005), we hypothesized that alcohol intake would be attenuated in D2R-OE_{NAcInd} mice. However, we did not observe a decrease in alcohol consumption during either CA or IA procedures. We found a generalized increase in fluid consumption in D2R-OE_{NAcInd} mice, which may be related to their higher activity as measured by hyperlocomotion. D2R upregulation resulted in increased alcohol consumption in the CA schedule, but did not alter intake in the IA schedule. The main difference between both models is that in the IA model, chronic cycling between periods of alcohol drinking and deprivation leads to enhanced escalation of consumption and thereby intoxicating blood ethanol concentrations. This is rarely observed using the CA model (Crabbe *et al*, 2011; Hwa *et al*, 2011). We suspect that this key aspect of the IA model may account for the lessened influence of the hyperingestive behavior over alcohol consumption in the IA model. Neither model, however, revealed a reduction in alcohol intake, arguing against a protective role of D2R overexpression on escalation of voluntary alcohol drinking. Although preference for ethanol in the IA procedure was significantly reduced in $D2R_{NAcInd}$

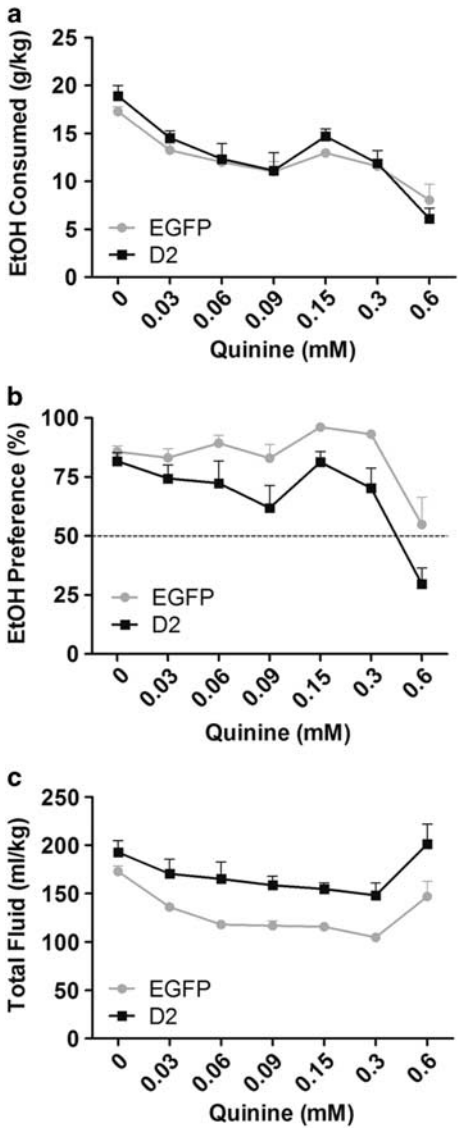

Figure 5 (a) After 10 IA sessions of 15% ethanol, quinine was faded into the ethanol solution beginning with concentrations that were aversive in alcohol-naïve mice (0.03–0.06 mM) up to 0.6 mM. Both groups showed a slow, but comparable decrease in their alcohol consumption with increasing quinine concentrations. (b) Although D2R-OE_{NAcInd} maintained a lower preference for alcohol $p < 0.05$ both groups were similarly resistant to reducing their preference with increasing quinine concentrations, eventually decreasing preference at the highest quinine dose. (c) Total fluid consumption was stable across quinine doses, but remained higher in the D2R-OE_{NAcInd} group ($p < 0.05$) ($F_{(1,14)} = 8.13$,).

mice, this decrease was driven by enhanced water intake, and not a reduction in alcohol intake. In line with this interpretation, in session 10 (Figure 4) where control and D2R-OE mice consume similar doses of total fluid (Figure 4d) and water (Supplementary Figure S2d), D2R-OE_{NAcInd} mice did not show decreased preference for alcohol.

Furthermore, both the D2R-OE_{NAcInd} and $EGFP_{NAcInd}$ mice were similarly sensitive to modulation of alcohol consumption and preference when the alcohol solution was adulterated with quinine. Consumption and preference shifted to the water bottle only at the highest dose of quinine (0.6 mM). This concentration was 10-fold higher than the concentrations needed to curtail their drinking behavior in the absence of alcohol (Figure 3g and j), in line with previous reports in C57BL6 mice with a history of chronic alcohol consumption (Lesscher *et al*, 2010). The comparable tolerance to a quinine-adulterated alcohol by both groups suggests that D2R overexpression in the NAc indirect pathway does not alter the impact of a negative outcome on elevated alcohol intake.

Our data seem at odds with the outcome of the adenovirus studies by Thanos *et al* (2001, 2004, 2005). There are several key differences in the objectives and design between these preceding studies and our work that may explain the apparent contradictions. First, the adenovirus experiments tested whether overexpression of D2Rs attenuates drinking after drinking behavior had already been established. In contrast, we used AAV to induce D2R overexpression *before* testing its effects on escalation of alcohol intake, in an effort to determine whether increased D2R levels confer protection against future drinking. It is conceivable that targeting D2R levels is only beneficial once drinking behavior or even alcohol dependence is established. Second, in the adenovirus experiments, D2Rs were overexpressed non-selectively in different cell types of the NAc, including the direct pathway that normally has only very limited expression of D2Rs (Bertran-Gonzalez *et al*, 2008). In contrast, using a Cre-LoxP- based strategy, we were able to restrict D2R overexpression to indirect pathway MSNs that express D2Rs. Given the lack of a protective effect by elevating D2R in this cell population, which includes MSNs that co-express D1Rs and D2Rs (Gerfen and Surmeier, 2011), it is possible that a D2R-based strategy aimed at attenuating drinking requires ectopic upregulation of D2Rs in direct pathway MSNs. This would be consistent with the classical model of basal ganglia circuitry and with observations in which inhibition of direct pathway MSNs impaired amphetamine sensitization (Ferguson *et al*, 2010) and cocaine place preference (Hikida *et al*, 2010). Third, the present experiments were designed to examine the long-term consequences of overexpressing D2R on drinking behavior. The time window of D2R overexpression in the adenovirus experiments was only several days, whereas AAV expression in our study is stable through at least 6 months after transduction (Supplementary Figure S1). We found that chronic D2R upregulation in adult alcohol-naive mice, however, does not prevent the escalation of alcohol drinking. This outcome suggests that ongoing, high D2R levels in the indirect pathway may not reduce vulnerability to this particular behavior. A lack of protective effect by cell type-specific D2R upregulation in this context does not rule out the possibility that our strategy could reduce vulnerability in D2R-deficient animals such as the high-alcohol preferring mouse strain (Bice *et al*, 2008). Moreover, high D2R levels in the indirect pathway may be protective against other drugs such as cocaine. However, a preliminary analysis of cocaine-related behaviors does not support this idea (Supplementary Figure S4).

Pharmacological studies using D2R agonists/antagonists in rodents or studies using D2R knockout mice have uncovered an involvement of D2Rs in a wide variety of

alcohol-related behaviors. In many cases, the findings support a protective role of increased D2R function (Levy *et al*, 1991; Ng and George, 1994; Hodge *et al*, 1997; Bulwa *et al*, 2011). However, other reports using D2R antagonists or D2R knockout mice, for instance, show a reduction in alcohol self-administration or preference (Rassnick *et al*, 1992; Samson *et al*, 1993; Hodge *et al*, 1997; Phillips *et al*, 1998; Thanos *et al*, 2005), or no difference at all (Linseman, 1990). These inconsistencies may stem from differences in the specific drinking models, dopamine agonists/antagonist doses, or routes of administration (NAc *vs* systemic), emphasizing the need for highly targeted genetic approaches for the study of D2R function.

Our viral approach has enabled us to test the specific consequences of D2R upregulation in indirect pathway MSNs of the NAc on locomotor activity and drinking behavior, thus overcoming some of the limitations outlined above. Although indirect pathway D2R upregulation did not prevent alcohol drinking behavior, a protective action may require overexpression of indirect pathway D2Rs in other striatal regions, such as the NAc shell or the dorsal striatum. Our findings also raise the possibility that the D2R alterations that are thought to influence alcohol dependence in humans may not be confined to the indirect pathway of the NAc. In addition to MSNs, D2Rs are also found in striatal cholinergic interneurons and dopamine neuron terminals, where they regulate key functions, including dopamine release (Bello *et al*, 2011; Cachope *et al*, 2012; Threlfell *et al*, 2012). Future studies may benefit from implementing approaches like the ones described here to systematically characterize whether increased D2R levels in other cell types reduce susceptibility to alcohol drinking.

FUNDING AND DISCLOSURE

This work was supported by NIH T32 fellowship (T32DA016224) to E.F.G, NIH/NIAAA fellowship (5F32AA022028) for M.C.S., NIH grants R01DA025036 and R01DA027460 to J.A.M., NIH grant R01AA019801 to N.L.H., NIH grants MH54137 and DA022413 to J.A.J, and NIH grant MH093672 and Columbia University start-up funds to C.K. The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Nicholas Grahame and Dr Diana Martinez for their insightful comments on the manuscript. We also thank Claire Sunha Choi and Jeremy Sherman for their valuable technical assistance in conducting the 2-bottle choice experiments.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)