Cellular/Molecular

The Dopamine D_3 Receptor Is Part of a Homeostatic Pathway Regulating Ethanol Consumption

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We recently identified a homeostatic pathway that inhibits ethanol intake. This protective pathway consists of the scaffolding protein RACK1 and brain-derived neurotrophic factor (BDNF). RACK1 translocates to the nucleus after exposure of neurons to ethanol and increases expression of BDNF (McGough et al., 2004). We also found that increasing the levels of BDNF via systemic administration of RACK1 expressed as a Tat-fusion protein (Tat–RACK1) reduces ethanol consumption, whereas reduction of BDNF levels augments this behavior (McGough et al., 2004). Based on these results, we hypothesized that activation of the BDNF receptor TrkB is necessary for the effects of BDNF on ethanol intake and that gene products downstream of BDNF negatively regulate ethanol consumption. Here, we show that inhibition of the BDNF receptor TrkB increases voluntary ethanol consumption in wild-type mice but not in mice lacking one copy of the BDNF gene (BDNF $^{+/-}$). We also find that increases in the levels of *BDNF*, mediated by ethanol or RACK1, lead to increased dorsal striatal levels of the dopamine *D_3 receptor* (*D_3R*), a gene downstream of BDNF, via activation of the TrkB receptor. Finally, we show that the Tat–RACK1-mediated reduction of ethanol consumption is attenuated by coinjection with either the Trk inhibitor K252a or the dopamine D_3R-prefering antagonist U-99194A [5,6-dimethoxy-2-(di-*n*-propylamino)indan], suggesting that activation of the BDNF pathway via RACK1 leads to increased expression of the dopamine D_3R, which in turn mediates the attenuation of ethanol consumption.

Key words: RACK1; BDNF; dopamine; D_3 receptor; alcohol; addiction

Introduction

We recently identified a homeostatic pathway that inhibits the behavioral effects of ethanol. This protective pathway consists of the scaffolding protein RACK1 and brain-derived neurotrophic factor (BDNF). We found that RACK1 translocates to the nucleus after exposure of neurons to ethanol and increases expression of BDNF (McGough et al., 2004). We also found that increasing the levels of BDNF via systemic administration of RACK1 expressed as a Tat-fusion protein (Tat–RACK1) reduces ethanol consumption and sensitization (McGough et al., 2004). In contrast, male BDNF $^{+/-}$ mice that have a 50% reduction in BDNF expression show a higher degree of ethanol sensitivity in several behavioral procedures; these include consumption after a period of abstinence, conditioned place preference (CPP) to ethanol, and ethanol-induced sensitization of locomotor activity (McGough et al., 2004). In agreement with a role for BDNF in the effects of ethanol, female BDNF $^{+/-}$ mice are reported to consume more ethanol than BDNF $^{+/+}$ mice (Hensler et al., 2003).

Here, we set out to determine whether ethanol consumption is negatively regulated by activation of the BDNF pathway and to identify a possible mechanism by which this regulation occurs. BDNF has been shown to induce the expression of the dopamine *D_3 receptor* (*D_3R*), *neuropeptide Y*, *preprodynorphin*, *cholecystokinin*, and *substance P* in the striatum (Croll et al., 1994; Guillin et al., 2001), and our previous findings revealed that ethanol self-administration increases *BDNF* expression in the dorsal, but not ventral, striatum (McGough et al., 2004). Interestingly, an increase in the expression and/or function of some of these gene products attenuates the effects of addictive drugs such as amphetamine, cocaine, and ethanol, whereas decreases in their expression potentiate sensitivity to the drugs (Xu et al., 1997; Carlezon et al., 1998; Thiele et al., 1998; Pilla et al., 1999). We therefore postulated that upregulation of one or more of these genes via the BDNF pathway contributes to the regulation of ethanol consumption.

In this study, we focused on the possible interaction between ethanol and the D_3R. Several studies suggest that activation of the D_3R reverses behavioral effects of ethanol. For example, administration of the D_2R/D_3R agonist 7-hydroxy-2(di-*n*-propylamino)tetralin decreases ethanol self-administration and preference in rats (Cohen et al., 1998), and the D_3R antagonist 5,6-

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dimethoxy-2-(di-*n*-propylamino)indan (U-99194A) increases ethanol-induced CPP (Boyce and Risinger, 2000). Therefore, we hypothesized that the RACK1/BDNF pathway regulates ethanol intake via the D_3R. Here, we show that the BDNF signaling cascade is activated during voluntary ethanol consumption, leading to increased levels of *D_3R*, and that BDNF-induced decreases in drinking are attenuated by D_3R blockade. Together, our findings suggest that the effect of activation of the RACK1/BDNF pathway on ethanol intake is mediated by the D_3R.

Materials and Methods

Reagents

The Trk kinase inhibitor K252a was purchased from Alomone Labs (Jerusalem, Israel). The dopamine D_3R-prefering antagonist U-99194A and the dopamine D_2R antagonist eticlopride were purchased from Sigma (St. Louis, MO). BDNF was purchased from R & D Systems (Minneapolis, MN). Primers for reverse transcription-PCR (RT-PCR) were synthesized by BioSource International (Camarillo, CA). Tat–RACK1 and Tat–cyclin-dependent kinase inhibitor 27 (KIP^{27}) were expressed in *Escherichia coli* and purified as described previously (Nagahara et al., 1998; He et al., 2002).

Animals

Male Sprague Dawley rats (3–4 weeks of age for slice biochemistry experiments) and male Long–Evans rats (200–250 g at the time of the surgery) were obtained from Harlan (Indianapolis, IN). C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). $BDNF^{+/-(J129ftm1Jae/C57BL/6)}$, generated as described by Ernfors et al. (1994), were also obtained from The Jackson Laboratory. Mixed pairs of heterozygous $BDNF^{+/-\ (J129ftm1Jae/C57BL/6)}$ hybrid male and female mice were used to generate heterozygote and wild-type ($BDNF^{+/+}$) littermate control mice. Mouse genotypes were determined by RT-PCR analysis of products derived from tail mRNA. Animals used in these studies were housed under a 12 h light/dark cycle, with lights on at 7:00 A.M. and food and water available *ad libitum*. All animal procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council (1996).

D_3R expression

D_3R expression after BDNF, Tat–RACK1, or ethanol treatment of striatal slices. Coronal striatal slices (250 μm) were prepared from 3- to 4-week-old male Sprague Dawley rats. Slices were maintained for at least 1 h in artificial CSF (aCSF) containing the following (in mM): 126 NaCl, 1.2 KCl, 1.2 NaH_2PO_4, 0.01 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose, saturated with 95%O_2/5%CO_2 at 25°C. After recovery, striatal slices were transferred to fresh aCSF and treated as described in the figure legends. After treatment, slices were washed extensively with aCSF and immediately frozen in liquid nitrogen for later RT-PCR, as described below. For each experiment, four slices per treatment were used, and each experiment was replicated three to six times (as indicated in figure legends). For all experiments, each animal contributed slices to each treatment.

Dopamine receptor expression after systemic Tat–RACK1 treatment or ethanol self-administration in vivo. In the experiment using injection of Tat-fusion proteins, adult C57BL/6J mice were injected intraperitoneally 3 h before the beginning of the dark cycle with saline or Tat–RACK1. Six hours later, brains were removed rapidly, and bilateral tissue punches of the striatum were frozen in liquid nitrogen for later homogenate preparation, as described below. In the experiment using voluntary ethanol intake, mice with continuous access to ethanol and water or water only (see below, Ethanol self-administration), were killed 3 h after the beginning of the dark cycle, brains were immediately removed, and bilateral tissue punches of the striatum were frozen in liquid nitrogen for later RT-PCR, as described below.

Dopamine receptor expression after dorsal striatal infusion of Tat–RACK1 in vivo. Adult Long–Evans rats were anesthetized continuously with isoflurane (Baxter Health Care Corporation, Deerfield, IL) and placed within a standard stereotaxic device. Tat–RACK1 (1 μl of a solution of 1 μM) was infused via a Hamilton 10 μl syringe (number 1701) into the dorsal striatum (anteroposterior, +1.2 mm; mediolateral, ±1.5 mm; dorsoventral, −4.5 mm; relative to bregma) over 2 min with the needle of the syringe kept in position for an additional 2 min. The subject remained anesthetized for 4 h, at which time the brain was taken. Striatal levels of the dopamine receptors were determined by RT-PCR as described below.

Preparation of homogenates and RT-PCR. Coronal striatal slices or dissected striata were homogenized in TRIzol (Invitrogen, Carlsbad, CA), and total RNA was isolated. The expression of *D_3R*, *D_2R*, the dopamine *D_1R*, and glycerol-3-phosphate dehydrogenase (*GPDH*) was analyzed by RT-PCR as described by McGough et al. (2004). The primers were designed as follows: *D_3R*: upstream, 5′-GGA GCA CAT AGA AGA CAA ACA ATA TCC CCA-3′ and downstream, 5′-CAA TGA CCA CCA TCT GGG TGG CCT TCT TCT-3′; *D_1R*: upstream, 5′-AGC CCT TTC CAG TAT GAG AGG AAG-3′ and downstream, 5′-TCT GGC AGT TCT TGG CAT GGA CTG-3′; and *D_2R*: upstream, 5′-TAG CAG CCG AGC TTT CAG AGC CAA-3′ and downstream, 5′-CTG AGT GGC TTT CTT CTC CTT CTG-3′.

Ethanol self-administration in mice

Voluntary ethanol intake in singly housed adult male C57BL/6J, $BDNF^{+/-}$, and $BDNF^{+/+}$ mice was established by placing a bottle containing 10% ethanol in tap water (v/v) on the home cage next to an identical bottle filled with tap water. The position of the bottles was switched every other day. Only subjects that consumed at least 40% of their total fluid volume as ethanol were included in these studies to ensure intake of pharmacologically significant amounts of ethanol. $BDNF^{+/-}$ and $BDNF^{+/+}$ F2 male mice used in the studies were 7–9 weeks of age at the beginning of ethanol exposure; C57BL/6J mice were 8–14 week of age. For studies on the effects of ethanol self-administration on *D_3R* expression, age-matched control mice experienced identical housing conditions and handling but were exposed to only water on the home cage.

Effects of the Trk inhibitor K252a on ethanol self-administration in mice. Voluntary ethanol intake protocol was as described above. After 4 weeks of drinking, the mice ($BDNF^{+/+}$ or $BDNF^{+/-}$) received an intraperitoneal injection of K252a (5 or 25 μg/kg) at 3:00 P.M.; ethanol and water intake were measured at 9:00 A.M. K252a was administered in an injection volume of 1 ml per 100 g of body weight.

Effects of Tat–RACK1 alone or in combination with K252a on ethanol self-administration in mice. Voluntary ethanol intake protocol was as described above. After 4 weeks of drinking, C57BL/6J mice received intraperitoneal injections of Tat–RACK1 (4 mg/kg), Tat–RACK1 and K252a (25 μg/kg), or saline at 3:00 P.M.; ethanol and water intake were measured at 9:00 A.M. the next day. Compounds were administered in injection volumes of 1 ml per 100 g of body weight.

Effects of Tat–RACK1 and dopamine receptor antagonists on ethanol self-administration in mice. Voluntary ethanol intake protocol was as described above. After 4 weeks of drinking, the mice (C57BL/6J) received intraperitoneal injections of Tat–RACK1 (2 mg/kg) or saline at 3:00 P.M. and subcutaneous injections of the D_3R-prefering antagonist U-99194A (20 mg/kg) (Gyertyan and Saghy, 2004) or saline, or intraperitoneal injections of the D_2R antagonist eticlopride (0.1 mg/kg) (Chausmer and Katz, 2001) or saline at 6:00 P.M.. Ethanol and water were made available again at 6:00 P.M., and intake was measured 4 h later at 10:00 P.M., so that the behavioral measurements were made at a time congruent with the *in vivo* biochemical measurements. Tat–RACK1, U-99194A, and eticlopride were administered in injection volumes of 1 ml per 100 g of body weight.

Operant ethanol self-administration in rats

Effect of Tat–RACK1 infusion into the dorsal striatum on ethanol operant self-administration. The operant self-administration procedure is as described previously (He et al., 2005) except that the rats were habituated to drink ethanol in their home cages, rather than in the self-administration chambers, beginning 1 week after surgery. After 3 weeks of exposure to ethanol in the home cage, the rats were trained 5 d a week in 60 min sessions for 2 months to self-administer a solution of 10% ethanol. A 0.1 ml drop of 10% ethanol was delivered after every third active lever response (fixed ratio-3).

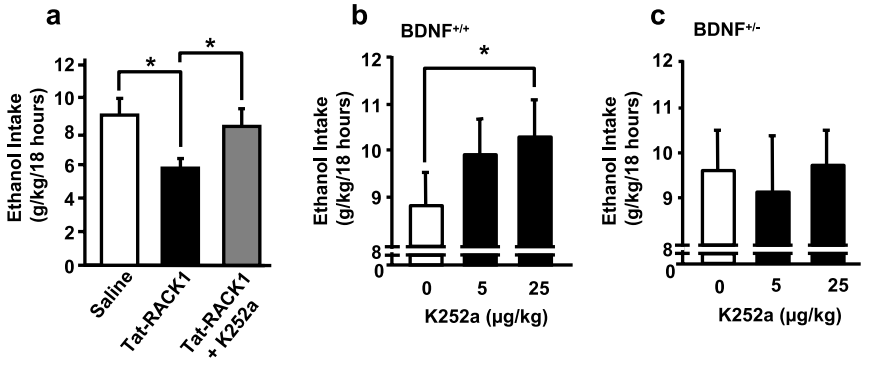

Figure 1. ***a***, K252a attenuates the Tat–RACK1 effect on voluntary ethanol intake in mice. Tat–RACK1 (4 mg/kg), Tat–RACK1 (4 mg/kg) plus K252a (25 μg/kg), or vehicle was administered at 3 P.M., and the amount of ethanol consumed overnight was recorded. Data are presented as mean ± SEM grams/kilogram of body weight. $n = 12$. *$p < 0.01$ compared with vehicle. ***b***, ***c***, Activation of the BDNF receptor TrkB regulates ethanol consumption. The Trk receptor inhibitor K252a (5 and 25 μg/kg) or vehicle was administered at 3 P.M., and the amount of ethanol consumed overnight was recorded. Data are presented as mean ± SEM grams/kilogram of body weight. ***b***, Inhibition of the Trk receptor increases ethanol intake in BDNF $^{+/+}$ mice. $n = 12$. *$p < 0.05$ compared with saline. ***c***, K252a has no effect on ethanol intake in BDNF $^{+/-}$ mice ($n = 9$).

Microinfusion of Tat–RACK1 into the dorsal striatum. Four hours before the test self-administration session, Tat–RACK1 (1 μl of a solution of 1 μM) or PBS (1 μl) was infused via a Hamilton 25 μl syringe (number 1702) into the dorsal striatum over 2 min with the injector kept in position in the cannula for an additional 2 min. Rats remained in the home cage until the beginning of the self-administration session. One week later, rats that received Tat–RACK1 in the previous week were tested after PBS administration and vice versa.

Surgery. Male Long–Evans rats (weighing 200–250 g) were anesthetized continuously with isoflurane (Baxter Health Care Corporation) during the surgery. Four holes were drilled for screws, and two other holes were drilled for the placement of the cannula (double cannula C235G-3.0; diameter, 22 ga, Plastics One, Roanoke, VA). The coordinates for the implantation were 1.2 mm anterior of bregma and 1.5 mm lateral of the medial suture. The cannulas (5.5 mm length under pedestal) were implanted carefully into the dorsal striatum (−4.5 mm from the skull surface) and fixed with dental cement.

Data analysis

Behavioral data were analyzed using ANOVA followed, when indicated by significant main effects of treatment, by Student's Newman–Keuls test. Alterations in D_3R expression were analyzed using Student's t test. Significance for all tests was set at $p < 0.05$.

Results

Activation of the BDNF receptor TrkB regulates ethanol consumption

We previously observed that systemic administration of RACK1 expressed as a Tat-fusion protein led to an increase in *BDNF* mRNA levels and to a reduction in ethanol consumption in a BDNF-dependent manner (McGough et al., 2004). We hypothesized that if the actions of Tat–RACK1 on ethanol self-administration are mediated via activation of the BDNF signaling cascade, then inhibition of the BDNF-mediated cascade should block the effects of Tat–RACK1 on ethanol intake. Activation of the BDNF-mediated signaling cascade is initiated via ligation of BDNF to its receptor, TrkB, resulting in autophosphorylation of the receptor and activation of downstream signaling cascades (Patapoutian and Reichardt, 2001). We therefore determined the effects of the Trk kinase inhibitor K252a (Tapley et al., 1992) on voluntary ethanol intake in C57BL/6J mice. As predicted, coinjection of Tat–RACK1 with K252a prevented the reduction in ethanol intake seen after Tat–RACK1 administration alone (Fig. 1*a*). Analysis of these data revealed a main effect of treatment ($F_{(2,22)} = 8.5$; $p < 0.003$), accounted for by a significant decrease in intake after Tat–RACK1 treatment ($p < 0.003$), but not Tat–RACK1 plus K252a ($p > 0.05$), compared with intake after saline injection. There was no effect of any treatment on water intake by these subjects ($F_{(2,22)} = 0.035$; $p = 0.97$). These results suggest that Tat–RACK1, when administered systemically, acts by activating the BDNF pathway.

We also observed that voluntary ethanol intake increases the expression of *BDNF*; however, decreasing the levels of BDNF increases the sensitivity of mice to the behavioral effects of ethanol (McGough et al., 2004). We hypothesized that the ethanol-mediated increase in dorsal striatal BDNF is part of a homeostatic pathway that counteracts the neurochemical systems responsible for the escalation and maintenance of ethanol consumption. If this is so, then inhibition of the BDNF signaling pathway should result in increased ethanol intake. To test this prediction, we tested the ability of the Trk receptor inhibitor K252a to enhance ethanol intake. Because K252a is not a specific inhibitor of TrkB but can also affect other Trk receptors, we conducted the experiment in BDNF $^{+/+}$ and BDNF $^{+/-}$ mice. As shown in Figure 1*b*, K252a increased ethanol consumption in BDNF $^{+/+}$ mice (main effect of treatment; $F_{(2,22)} = 4.03$; $p < 0.03$) at 25 μg/kg ($p < 0.03$), with a nearly significant increase at 5 μg/kg ($p = 0.06$). Water consumption was not affected ($F_{(2,22)} = 2.20$; $p = 0.14$). However, increased ethanol consumption was not observed in BDNF $^{+/-}$ mice ($F_{(2,16)} = 0.96$; $p = 0.41$) (Fig. 1*c*).

Activation of the BDNF pathway via RACK1 leads to increased D_3R expression

Subsequently, we tested the hypothesis that the activation of the BDNF signaling cascade leads to the upregulation of downstream genes, specifically the dopamine D_3R, which in turn regulates ethanol consumption. The expression of the D_3R has been shown to be upregulated via BDNF in the striatum (Guillin et al., 2001), and we found that ethanol and Tat–RACK1 increase *BDNF* expression in the dorsal striatum (McGough et al., 2004). To test the possibility that activation of the BDNF pathway in the dorsal striatum via RACK1 leads to an increase in the expression of the D_3R, we first confirmed that BDNF increases dorsal striatal expression of the D_3R. As can be seen in Figure 2*a*, incubation of striatal slices with BDNF increased the expression of the D_3R, and this effect was blocked by the Trk inhibitor K252a. Next, we set out to test whether increasing the protein levels of RACK1 would increase the expression of the D_3R. As shown in Figure 2*b*, incubation of striatal slices with Tat–RACK1 increased the expression of D_3R with the maximal effect detected after 4 h of Tat–RACK1 incubation. We reasoned that if the increase in mRNA levels in the presence of Tat–RACK1 is mediated via activation of the BDNF pathway, then the increase in D_3R should not be detected when BDNF signaling is inhibited. We found that the Tat–RACK1-induced increase in D_3R expression is blocked by pretreatment with the Trk inhibitor K252a (Fig. 2*c*).

Importantly, systemic injection of Tat–RACK1, which increases *BDNF* expression in the dorsal striatum (McGough et al., 2004), also led to an elevation of D_3R mRNA levels in the same brain region (Fig. 3*a*). This increase is specifically induced by Tat–RACK1, because the unrelated Tat-fusion protein Tat–KIP^{27} had no effect (Fig. 3*a*).

Finally, this Tat–RACK1-mediated increase is selective for D_3R, because the levels of two other dopamine receptors, D_1R and D_2R, were not altered (Fig. 3*b*).

To further confirm that Tat–RACK1 increases D_3R expression in the dorsal striatum, Tat–RACK1 was infused directly into the dorsal striatum of rats. Tat–RACK1 led to a significant elevation in D_3R mRNA ($p < 0.01$) compared with PBS (Fig. 3*c*), and the levels of D_1R and D_2R were unchanged (Fig. 3*d*).

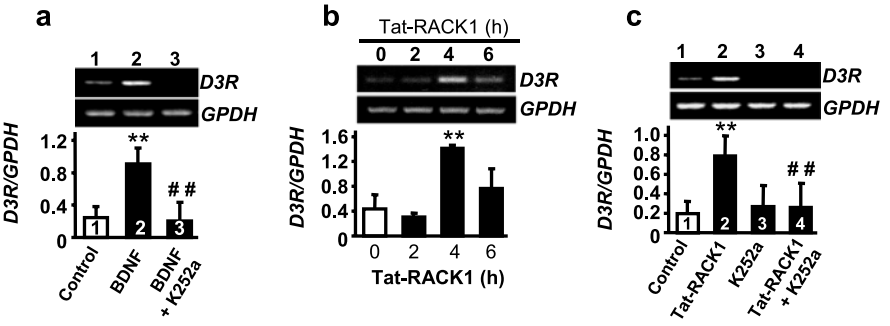

Figure 2. Tat-RACK1 and BDNF induce D_3R expression in the striatum via activation of the TrkB receptor. ***a***, Striatal slices dissected from five rats were preincubated without (lanes 1 and 2) or with 200 nM K252a (lane 3) for 30 min before addition of 50 ng/ml BDNF (lanes 2 and 3) for 1 h. D_3R expression was analyzed by RT-PCR with *GPDH* control. The histogram depicts the mean ratios of D_3R to *GPDH* ± SD. $n = 5$. $^{**}p < 0.01$ compared with control, or $^{**}p < 0.01$ comparing BDNF plus K252a with BDNF alone. ***b***, Striatal slices dissected from four rats were treated with 1 μM Tat–RACK1 for the indicated times. D_3R expression was analyzed by RT-PCR with *GPDH* control. The histogram depicts the mean ratios of D_3R to *GPDH* ± SD. $n = 3$. ***c***, Striatal slices dissected from 10 rats were preincubated without or with 200 nM K252a (lanes 3 and 4) for 30 min before addition of 1 μM Tat–RACK1 (lanes 2 and 4) for 4 h. D_3R expression was analyzed by RT-PCR with *GPDH* control. The histogram depicts the mean ratios of D_3R to *GPDH* ± SD. $n = 6$. $^{**}p < 0.01$ compared with control, or $^{\#\#}p < 0.01$ comparing Tat-RACK1 plus K252a with Tat-RACK1 alone.

Intradorsal striatum injection of Tat–RACK1 reduces ethanol consumption

We found that systemic injection of Tat–RACK1 induces a decrease in ethanol consumption (McGough et al., 2004) (Fig. 1*a*). To test the possibility that Tat–RACK1-mediated reduction of ethanol consumption is localized to the dorsal striatum, we microinjected the protein into the dorsal striatum of rats trained to self-administer ethanol. We found that Tat–RACK1 infusion significantly decreased responding for ethanol compared with responding after infusion of PBS (Fig. 3*e*). Analysis of these data (two-way repeated measures ANOVA) revealed a main effect of treatment ($F_{(1,10)} = 7.33$; $p < 0.05$) and of lever ($F_{(1,10)} = 24.36$; $p < 0.001$) but not a significant treatment-by-lever interaction. Planned comparisons found that Tat–RACK1 infusion significantly decreased active lever responding ($p < 0.02$) but not inactive lever responding ($p = 0.20$). Thus, injection of Tat–RACK1 into the dorsal striatum reduces ethanol consumption in an operant self-administration paradigm.

Ethanol exposure results in increased D_3R expression via BDNF signaling

We found that ethanol increases the expression of *BDNF* and that activation of the BDNF pathway regulates ethanol consumption (McGough et al., 2004) (Fig. 1*b*,*c*). Therefore, if D_3R is a downstream target of this homeostatic pathway, then ethanol exposure should also increase the expression of the receptor. We found that 3 h of incubation with ethanol induced a significant increase in D_3R expression in striatal slices (Fig. 4*a*). Next, we tested whether the increase in D_3R expression is mediated by activation of the BDNF pathway. As predicted, the ethanol-mediated increase in D_3R expression (Fig. 4*b*, lane 1 vs 2) was blocked by pretreatment of slices with K252a (Fig. 4*b*, lane 2 vs 4), whereas incubation with the inhibitor alone (Fig. 4*b*, lane 3) had no effect on the expression of this gene.

In agreement with our results obtained in striatal slices, we found that voluntary ethanol intake (averaging 4.95 ± 0.56 g/kg during the 3 h between the start of the dark cycle and the time the mice were killed) was associated with a significant increase in the expression of D_3R (Fig. 4*c*) in the dorsal striatum, compared with ethanol-naive control mice, without altering the expression of D_1R and D_2R (Fig. 4*d*).

Systemic administration of Tat–RACK1 reduces ethanol consumption via activation of the D_3R

Based on the results thus far, we hypothesized that the D_3R is part of the RACK1/BDNF pathway that regulates voluntary ethanol consumption. To test this hypothesis, we compared the levels of ethanol intake in mice treated with vehicle to mice treated with the D_3R-preferring antagonist U-99194A (Waters et al., 1993; Audinot et al., 1998). We also tested whether U-99194A would attenuate the reduction in ethanol consumption after administration of Tat–RACK1 (Fig. 5*a*). Analysis of these data revealed a main effect of treatment ($F_{(3,20)} = 11.16$; $p < 0.001$). We found that administration of the U-99194A resulted in a small increase in ethanol consumption; however, this increase did not attain statistical significance when compared with the untreated control group ($p = 0.12$) (Fig. 5*a*). Tat–RACK1 reduced ethanol consumption as compared with saline treatment ($p < 0.05$); however, administration of U-99194A attenuated the Tat–RACK1-induced decrease in ethanol consumption ($p < 0.05$) (Fig. 5*a*). Analysis of water intake found a significant effect of treatment ($F_{(3,20)} = 9.68$; $p < 0.001$), which was accounted for by an increase in water intake by the saline-treated group compared with all other treatment groups ($p < 0.001$); importantly, there was no difference in water intake among the mice receiving Tat–RACK1 or Tat–RACK1 plus U-99194A or U-99194A alone.

In contrast to the effect of the D_3R-preferring antagonist U-99194A, injection of the D_2R antagonist eticlopride did not attenuate the decrease in ethanol consumption induced by Tat–RACK1 (Fig. 5*b*). Analysis of the data shows a main effect of treatment ($F_{(3,21)} = 4.84$; $p < 0.01$). Tat–RACK1 decreased ethanol intake ($p < 0.05$), and this decrease was not blocked by coadministration of eticlopride (saline vs Tat–RACK1 plus eticlopride; $p < 0.05$). Finally, eticlopride alone did not change ethanol consumption compared with saline injection ($p = 0.26$), and there was no effect of Tat–RACK1 and/or eticlopride on water intake ($F_{(3,21)} = 0.96$; $p = 0.43$).

Discussion

Previously, we found that RACK1 and BDNF are part of a regulatory pathway that controls voluntary ethanol consumption (McGough et al., 2004). The current study was designed to determine whether activation of the BDNF receptor TrkB is necessary for the regulation of ethanol consumption by the RACK1/BDNF pathway and whether the D_3R is a downstream component of this cascade. We found that inhibition of the TrkB receptor increases ethanol consumption. We also showed that the reduction of ethanol consumption mediated by Tat–RACK1 requires the BDNF

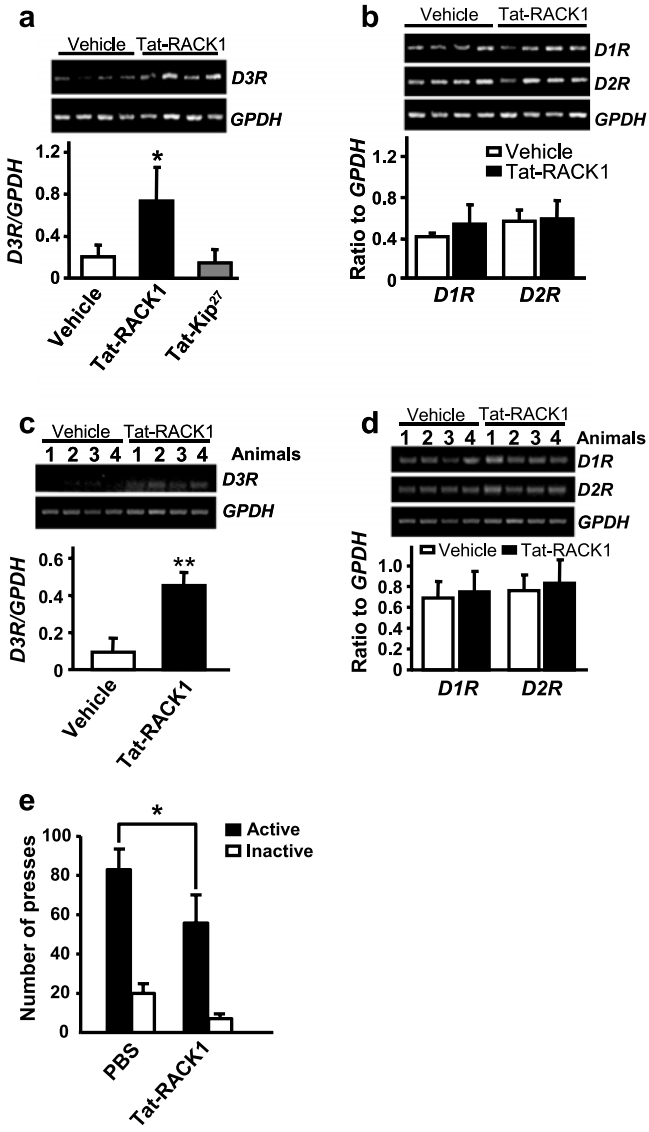

Figure 3. ***a***, ***b***, Tat–RACK1 increases D_3R expression in striatum *in vivo*. Six hours after intraperitoneal injection of vehicle or Tat–RACK1 (4 mg/kg), bilateral tissue punches of the striatum were taken and homogenized for RNA isolation as described in Materials and Methods. Expression of D_3R (***a***), D_1R, and D_2R (***b***) were analyzed by RT-PCR. The histograms depict the mean ratios of D_3R, D_1R, or D_2R to *GPDH* (±SD). $n = 12$ (4 mice used per treatment). $^*p < 0.05$ compared with vehicle. ***c***, Microinjection of Tat–RACK1 into striatum increases D_3R expression *in vivo*. Four rats were used for injection with PBS (1 μl per side) into one side of the dorsal striatum and Tat–RACK1 (1 μM, 1 μl per side) into the opposite side. Four hours after the microinjection, bilateral tissue punches of the striatum were dissected and homogenized for RNA isolation. Expression of D_3R (***c***), D_1R, and D_2R (***d***) were analyzed by RT-PCR. The histogram depicts the mean ratios of D_3R or D_1R, D_2R to *GPDH* ± SD. $n = 4$. $^{**}p < 0.01$ compared with vehicle. ***e***, Intradorsal striatum injection of Tat–RACK1 reduces ethanol consumption in rats. PBS (1 μl per side) or Tat–RACK1 (1 μM, 1 μl per side) was administered at 9:00 A.M. The rats were tested in the self-administration chambers at 1:00 P.M. for 1 h. The data are expressed in mean ± SEM of number of presses in 1 h. $n = 11$. $^*p < 0.05$ compared with PBS.

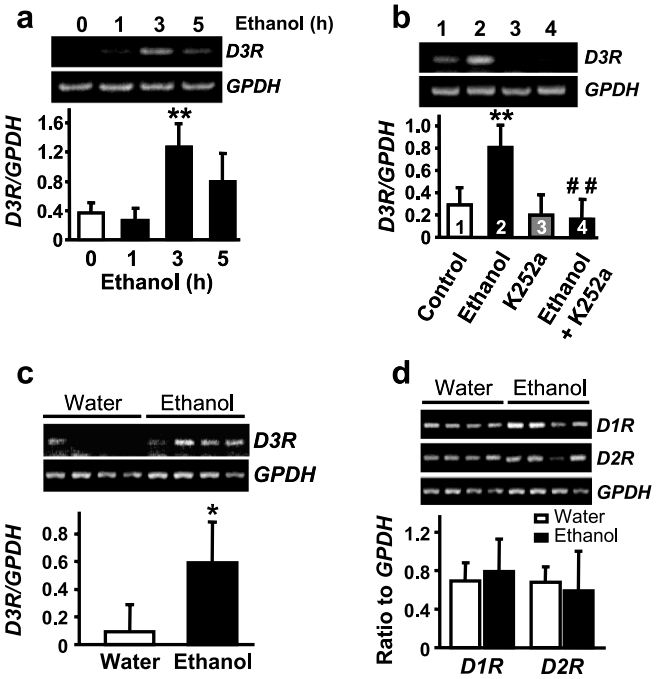

Figure 4. ***a***, ***b***, Ethanol induces D_3R expression in the striatum via activation of the TrkB receptor. ***a***, Striatal slices dissected from five rats were treated with 100 mM ethanol for the indicated times. D_3R expression was analyzed by RT-PCR with *GPDH* control. The histogram depicts the mean ± SD ratios of D_3R to *GPDH* from triplicate experiments ($n = 3$). ***b***, Striatal slices dissected from nine rats were preincubated without or with 200 nM K252a (lanes 3 and 4) for 30 min before the addition of 100 mM (lanes 2 and 4) ethanol for 3 h. D_3R expression was analyzed by RT-PCR with *GPDH* control. The histogram depicts the mean ± SD ratios of D_3R to *GPDH*. $n = 6$. $^{**}p < 0.01$ compared with control, or $^{\#\#}p < 0.01$ comparing ethanol plus K252a with ethanol alone. ***c***, ***d***, Ethanol self-administration increases D_3R expression in the striatum *in vivo*. C57BL/6J mice were allowed continuous access to ethanol for 4 weeks using the two-bottle choice procedure as described in Materials and Methods (ethanol group). Age-matched control mice (water group) were exposed to water only for the same time period. Three hours after the start of the dark cycle, bilateral tissue punches of the striatum were taken and homogenized for RNA isolation. Expression of D_3R (***c***), D_1R, and D_2R (***d***) were analyzed by RT-PCR. The histogram depicts the mean ratios of D_3R, D_1R, or D_2R to *GPDH*, ± SD. $n = 8$ (4 mice per group). $^*p < 0.05$ compared with the water group.

signaling pathway. We observed that both ethanol and Tat–RACK1 increase D_3R expression via activation of the BDNF signaling pathway, in striatal slices and in the dorsal striatum *in vivo*, and showed that the reduction of ethanol intake by Tat–RACK1 is inhibited in the presence of the D_3R-preferring antagonist U-99194A.

Based on these results, we present the following model (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Acute exposure to ethanol or transduction of recombinant Tat–RACK1 increases the expression of *BDNF* in the dorsal striatum. Secreted BDNF then activates the BDNF receptor TrkB, leading to the activation of a signaling cascade that induces expression of the dopamine D_3R. Activation of the D_3R, in turn, negatively regulates ethanol intake.

Ethanol- and Tat–RACK1-induced increases in D_3R expression are mediated via activation of the Trk receptor

We found that ethanol exposure or Tat–RACK1 treatment resulted in an increase in the expression of D_3R both *in vitro* and *in vivo*, and this increase was specific for D_3R, because the levels of the dopamine receptor subtypes D_1R and D_2R were unchanged by the treatments.

Because the increase in D_3R expression by ethanol or Tat–RACK1 was prevented by the Trk inhibitor K252a, we conclude that this increase requires activation of the BDNF signaling pathway. BDNF, through binding to the receptor tyrosine kinase TrkB, activates the mitogen-activated protein kinase (MAPK) signaling pathway (for review, see Pierce and Bari, 2001; Huang and Reichardt, 2003), leading to altered gene transcription (Patapoutian and Reichardt, 2001). The MAPK pathway activates transcription factors such as the cAMP response element (CRE)

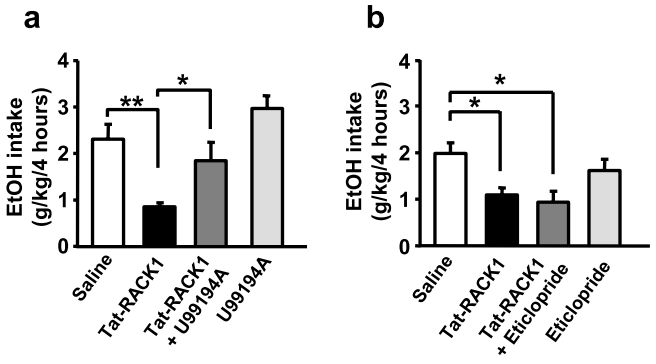

Figure 5. The D_3R-preferring antagonist U-99194A, but not the D_2R antagonist eticlopride, reduces the effect of Tat–RACK1 on ethanol (EtOH) consumption in mice. Tat–RACK1 or saline was administered at 3:00 P.M. followed at 6:00 P.M. by injection of the dopaminergic antagonists. The amount of ethanol consumed in 4 h between 6:00 and 10:00 P.M. was recorded. Data are presented as mean ± SEM grams/kilogram of body weight. ***a***, The dopamine D_3R antagonist U-99194A (20 mg/kg) attenuates the ability of Tat–RACK1 (2 mg/kg) to decrease drinking (saline, $n = 5$; Tat–RACK1, $n = 7$; Tat–RACK1 plus U-99194A, $n = 6$; U-99194A, $n = 6$). $^*p < 0.05$ compared with saline; $^{**}p < 0.01$ compared with Tat-RACK1 alone. ***b***, The significant decrease induced by Tat–RACK1 (2 mg/kg) injection is not altered by eticlopride (0.1 mg/kg) injection (saline, $n = 6$; Tat–RACK1, $n = 6$; Tat–RACK1 plus eticlopride, $n = 7$; eticlopride, $n = 6$). $^*p < 0.05$ compared with saline.

binding protein (CREB) (Finkbeiner et al., 1997; Pizzorusso et al., 2000); activation of CREB results in transcription of CRE-response genes (Gaiddon et al., 1996). The mechanism by which RACK1/BDNF increases *D_3R* transcription still remains to be demonstrated. However, as the *D_3R* promoter region contains a CRE element (D'Souza et al., 2001), it is plausible that BDNF upregulates *D_3R* expression via a MAPK-dependent activation of CREB.

The RACK1/BDNF pathway increases the expression of the *D_3R* in the dorsal striatum

The basal levels of *BDNF* expression in the dorsal striatum are low (Kawamoto et al., 1996) and are increased by ethanol or Tat–RACK1 treatment (McGough et al., 2004). Interestingly, the basal levels of *D_3R* expression in the dorsal striatum also are low (Diaz et al., 2000), and, as we show in this study, also are increased in response to ethanol and Tat–RACK1. To date, no other studies have reported changes in *D_3R* expression in the striatum after ethanol treatment. However, a few studies have reported changes after treatment with other drugs of abuse. Spangler et al. (2003) found that chronic morphine injection increased *D_3R* expression in the dorsal striatum, as well as the substantia nigra/ventral tegmental area. In addition, D_3R receptor number in the ventromedial and ventrolateral regions of the dorsal striatum was increased after acute cocaine injection 31–32 d after termination of cocaine self-administration (Neisewander et al., 2004).

D_3R expression has also been reported to be upregulated by exposure to other drugs of abuse in the neighboring striatal region, the nucleus accumbens (Le Foll et al., 2003; Neisewander et al., 2004). An increase in *D_3R* levels has been detected in the shell of the nucleus accumbens, but not in either the nucleus accumbens core or the dorsal striatum, after sensitization to nicotine (Le Foll et al., 2003). Also, acute injection of cocaine is reported to increase *D_3R* expression in the nucleus accumbens shell (Le Foll et al., 2005). Previous studies suggest that the drug-induced increases in *D_3R* expression in the accumbens depend on BDNF derived from cortical sources (Guillin et al., 2001; Le Foll et al., 2005). In contrast, we find that ethanol induces increases in both *BDNF* (McGough et al., 2004) and *D_3R* (present study) mRNA within the dorsal striatum itself. These region-specific effects could be because of differences in RACK1 compartmentalization and thus function in different brain structures (Yaka et al., 2003) including the dorsal versus ventral striatum (Phamluong and Ron, 2005), such that the cocaine- and nicotine-induced changes in *D_3R* expression in the accumbens may not be mediated by the RACK1/BDNF regulatory pathway. Certainly, it will be of great interest to test this possibility and to determine whether the dorsal striatal RACK1/BDNF/D_3R pathway described here is also activated by other drugs of abuse.

The RACK1/BDNF pathway controls ethanol consumption via the dopamine D_3R

Our results suggest that the reduction in ethanol consumption via the RACK1/BDNF pathway is mediated by increases in the levels of the D_3R in the dorsal striatum. We draw this conclusion although the antagonists we used to block the D_3R and D_2R, respectively, have some receptor cross-reactivity. Although we cannot definitively conclude that the effect of U-99194A is solely via the D_3R, the selectivity of U-99194A is reported to be higher for D_3R compared with D_2R (Franklin et al., 1998; Boulay et al., 1999; Gyertyan and Saghy, 2004). Likewise, the D_2R antagonist eticlopride is reported to have higher selectivity for D_2R than for D_3R (Tang et al., 1994). Together with the findings that ethanol and Tat–RACK1 increase only *D_3R* but not *D_2R* or *D_1R* levels, the hypothesis that the D_3R is an important mediator of the RACK1/BDNF pathway is the most parsimonious explanation of the findings to date.

Previous findings of the effects of systemic injection of D_3R agonists and antagonists are mixed, with some studies indicating that D_3R receptor activation decreases ethanol intake (Silvestre et al., 1996; Cohen et al., 1998), whereas others find the opposite (Thanos et al., 2005). It is possible that the effects observed here are specific to increases in the dopamine D_3R in the dorsal striatum and, for the reasons mentioned above, that D_3R activation in the dorsal striatum may have different behavioral effects than D_3R activation in other brain regions, such as the nucleus accumbens.

It is yet to be determined how increasing the levels of the *D_3R* leads to a reduction in ethanol-mediated behavior. The effectiveness of the D_3R-preferring antagonist suggests that endogenous dopamine activity at the D_3R contributes to the reduction in drinking. Ethanol in low doses is reported to increase the firing of dopaminergic neurons, including those of the substantia nigra (Mereu et al., 1984), whereas effects on dopamine in the dorsal striatum itself are mixed, with increases, decreases, and no effect reported (Imperato and Di Chiara, 1986; Blanchard et al., 1993; Budygin et al., 2001). An intriguing possibility is that ethanol exposure simultaneously increases dopamine release and D_3R levels in the dorsal striatum and that the activation of the D_3R by dopamine controls ethanol intake. This possibility is in line with previous studies that suggest a modulatory role for the D_3R in regulating the activities of the D_1R and D_2R (Xu et al., 1997). Xu et al. (1997) reported that modulation of locomotor activity induced by either a D_1R or D_2R agonist is not different in $D_3R^{-/-}$ mice compared with $D_3R^{+/+}$. However, when the agonists are given together, $D_3R^{-/-}$ mice exhibit increased locomotor activity. In addition, D_1R and D_2R agonist coadministration produces greater ERK phosphorylation in the striatum of $D_3R^{-/-}$ mice than in wild-type mice (Zhang et al., 2004). The authors conclude that activation of the D_3R downregulates excessive activation of the D_1R and D_2R. Thus, changing the levels of expression of the

D_3R may modulate the behavioral effects of dopamine mediated by the other receptor subtypes and, hence, alter behavior.

Other pathways that may contribute to the regulation of ethanol consumption via BDNF

The current studies indicate that the D_3R may be part of a BDNF pathway that regulates ethanol consumption. However, it is plausible that BDNF-mediated induction of genes other than *D_3R* contributes to the regulation of consumption. For example, BDNF infusion into the dorsal striatum results in increased expression of multiple additional genes, including *neuropeptide Y*, *preprodynorphin*, and *cholecystokinin* (Croll et al., 1994), and activation of one or more of these pathways may also contribute to the regulation of ethanol consumption.

Summary

In summary, our results show that regulation of ethanol intake is mediated by activation of the BDNF pathway, followed by a specific increase in *D_3R* expression in the dorsal striatum. Thus, we have identified a new role for the D_3R. Our results suggest that this dopamine receptor plays a key regulatory role in a homeostatic pathway that controls ethanol consumption. We hypothesize that a malfunction of this regulatory pathway may lead to ethanol addiction. Therefore, agents that activate this signaling cascade may be of great use in the treatment of alcohol addiction.

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