

GDNF is a fast-acting potent inhibitor of alcohol consumption and relapse

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Previously, we demonstrated that the action of the natural alkaloid, ibogaine, to reduce alcohol (ethanol) consumption is mediated by the glial cell line-derived neurotrophic factor (GDNF) in the ventral tegmental area (VTA). Here we set out to test the actions of GDNF in the VTA on ethanol-drinking behaviors. We found that GDNF infusion very rapidly and dose-dependently reduced rat ethanol, but not sucrose, operant self-administration. A GDNF-mediated decrease in ethanol consumption was also observed in rats with a history of high voluntary ethanol intake. We found that the action of GDNF on ethanol consumption was specific to the VTA as infusion of the growth factor into the neighboring substantia nigra did not affect operant responses for ethanol. We further show that intra-VTA GDNF administration rapidly activated the MAPK signaling pathway in the VTA and that inhibition of the MAPK pathway in the VTA blocked the reduction of ethanol self-administration by GDNF. Importantly, we demonstrate that GDNF infused into the VTA alters rats' responses in a model of relapse. Specifically, GDNF application blocked reacquisition of ethanol self-administration after extinction. Together, these results suggest that GDNF, via activation of the MAPK pathway, is a fast-acting selective agent to reduce the motivation to consume and seek alcohol.

addiction | growth factor | self-administration

Glial cell line-derived neurotrophic factor (GDNF) is an essential growth factor for the development of the kidneys and spinal cord motoneurons and exerts a wide range of effects on peripheral and central neurons (1). GDNF is also a potent trophic factor for midbrain dopaminergic neurons *in vitro* (2), but the function of GDNF in the development and maintenance of dopaminergic neurons *in vivo* remains unclear (3–6). However, strong evidence supports an important neurorestorative role of exogenously applied (7, 8) and endogenous (9) GDNF after lesion of the nigrostriatal system. GDNF acts through a multi-component receptor system including the glycosyl-phosphatidylinositol-linked GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) and the tyrosine kinase receptor Ret (1). Ligand of GDNF to GFR $\alpha 1$ leads to the recruitment and activation of Ret and to the consequent activation of the MAPK, phosphoinositide 3-kinase (PI3K), and phospholipase C γ (PLC γ) pathways (1). In addition, Src family tyrosine kinases have been implicated in GDNF-mediated functions mainly via a Ret-independent mechanism (10).

GFR $\alpha 1$ and Ret are highly expressed in the midbrain ventral tegmental area (VTA) (11, 12), a brain region that is a critical component of the neural circuitry involved in drug- and alcohol-seeking behavior (13–15). Moreover, VTA dopaminergic neurons are selectively vulnerable to some neuroadaptations induced by repeated exposure to drugs of abuse and ethanol (16, 17). Interestingly, a role for GDNF in addiction has been suggested based on evidence acquired from the examination of a variety of drugs of abuse (18). For example, repeated administration of cocaine and morphine decreases Ret phosphorylation (i.e., activity) in the VTA (19), whereas phencyclidine administration was found to increase GDNF expression in the VTA and the substantia nigra (20). In addition, administration of GDNF into the VTA blocks biochemical adaptations to

cocaine and morphine exposure (19). Furthermore, heterozygous GDNF knockout mice (Het) are more vulnerable to morphine- and cocaine-induced psychomotor sensitization than their wild-type (WT) littermates (19, 21). The GDNF Het mice also exhibit increased sensitivity to cocaine place conditioning (19) and to acquisition and reinstatement of methamphetamine self-administration compared with the WT mice (22). Conversely, intra-VTA infusion of GDNF reduces cocaine place conditioning (19), and sustained administration of GDNF in the striatum impedes acquisition of cocaine self-administration (23, 24). In addition, Niwa *et al.* (25) recently reported that increasing GDNF expression in the brain blocks methamphetamine place conditioning and psychomotor sensitization. Finally, we previously showed that the decrease in ethanol self-administration induced by the natural alkaloid ibogaine is mediated by the up-regulation of GDNF and activation of its signaling pathway in the VTA (26). Interestingly, we also found a reduction of ethanol self-administration after intra-VTA injection of a single dose of GDNF (26). More recently, we showed that the sustained actions of ibogaine are mediated via an autoregulatory positive feedback loop in which GDNF triggers its own expression (27). Taken together, these data suggest that stimulation of the GDNF pathway in the mesolimbic system may be a valuable strategy to combat alcoholism. Therefore, we set out to characterize the ability of GDNF in the VTA to regulate alcohol-drinking behavior and to identify a molecular mechanism that mediates its action.

Results

Intra-VTA Microinjection of GDNF Rapidly Decreases Ethanol Self-Administration. First, we tested the effect of intra-VTA administration of GDNF on rat operant self-administration of a 10% ethanol solution (28). We found that GDNF infused into the VTA 10 min before the test session dose-dependently decreased responding at the ethanol lever (Fig. 1A). GDNF infused into the VTA 3 h before the beginning of the test session was also effective in decreasing lever presses for ethanol (Fig. 1B). Analysis of cumulative active lever-press responding within the test session after the microinjection of PBS and the highest dose of GDNF (10 μ g per side) revealed a reduction in the frequency of lever-pressing bouts and an early termination of drinking, with no change in the initiation of lever pressing for ethanol [supporting information (SI) Fig. S1 and Table S1]. Next we examined the effect of GDNF on ethanol self-administration in rats with a history of high voluntary ethanol consumption (29) (Fig.

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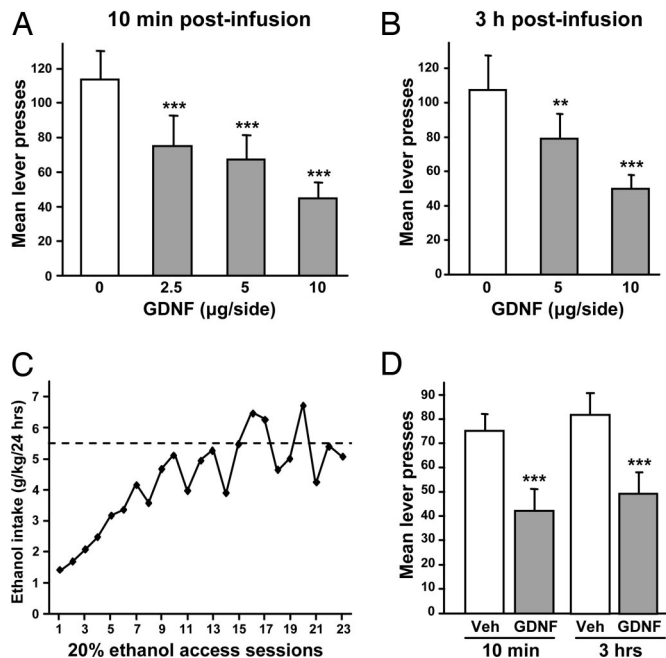


Fig. 1. Ten minutes and 3 h post infusion of GDNF in the VTA decreases operant ethanol self-administration. (A) Mean \pm SEM number of lever presses in 1 h after GDNF microinjection into the VTA (0, 2.5, 5, or 10 μ g per side) 10 min before the self-administration session. Two-way ANOVA with repeated measures showed significant effects of lever [$F_{(1,24)} = 27.08, P < 0.001$] and treatment [$F_{(3,24)} = 9.96, P < 0.001$] and a significant interaction between both factors [$F_{(3,24)} = 9.16, P < 0.001$] ($n = 10$). (B) Mean \pm SEM number of lever presses in 1 h after GDNF microinjection into the VTA (0, 5, or 10 μ g per side) 3 h before the self-administration session [main effect of lever: $F_{(1,16)} = 13.28, P < 0.001$; treatment: $F_{(2,16)} = 26.15, P < 0.001$; and a significant interaction: $F_{(2,16)} = 11.80, P < 0.001$] ($n = 10$). (C) Mean \pm SEM of ethanol intake during acquisition of voluntary ethanol consumption of a 20% ethanol solution in an intermittent-access (24 h on/24 h off) two-bottle choice paradigm. After 13–14 sessions, rats maintained drinking levels of 5.5 ± 1.5 g/kg in 24 h, with a consumption of 1–1.5 g/kg during the first 30 min of access to ethanol ($n = 8$). (D) Mean \pm SEM number of lever presses in 30 min for a 20% ethanol solution after acquisition of ethanol drinking in the intermittent-access two-bottle choice paradigm following intra-VTA infusion of GDNF (0 or 10 μ g per side) before the session [10 min before session, main effect of lever: $F_{(1,7)} = 70.56, P < 0.001$; treatment: $F_{(1,7)} = 13.79, P < 0.01$; and a significant interaction: $F_{(1,7)} = 12.26, P < 0.01$; 3 h before session main effect of lever: $F_{(1,7)} = 37.81, P < 0.001$; main effect of treatment: $F_{(1,7)} = 10.85, P < 0.02$; significant interaction: $F_{(1,7)} = 7.4, P < 0.05$] ($n = 8$). **, $P < 0.01$; ***, $P < 0.001$ (compared with PBS injection).

1C and Fig. S2), which approximates the ethanol intake observed in alcohol-preferring rats (30). After 24 drinking sessions, rats were trained to lever-press for a 20% ethanol solution. Importantly, infusion of GDNF (10 μ g per side) into the VTA 10 min and 3 h before the session also reduced ethanol self-administration in this paradigm (Fig. 1D). Taken together, these results suggest that GDNF-mediated reduction of operant ethanol self-administration is rapid and persistent.

Microinjection of GDNF into the Substantia Nigra Pars Compacta (SNc) Does Not Decrease Ethanol Self-Administration. To determine the site-specificity of GDNF's action, we infused the growth factor into the neighboring midbrain dopaminergic region, the SNc. As shown in Fig. 2A, infusion of GDNF into the SNc did not alter lever-press responding for ethanol, suggesting that the decrease in ethanol self-administration observed after microinjection of GDNF is specific for the VTA.

Intra-VTA Microinjection of GDNF Does Not Decrease Sucrose Self-Administration. Next we determined the specificity of GDNF's actions by examining its ability to attenuate self-administration

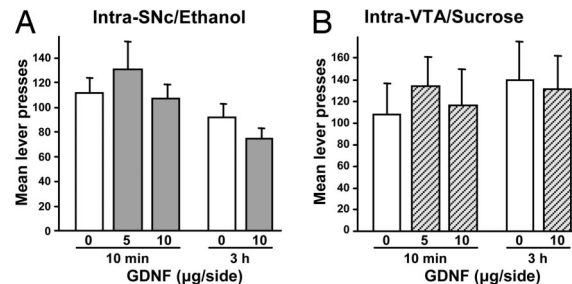


Fig. 2. Intra-substantia nigra injections of GDNF do not affect operant self-administration, and intra-VTA injections of GDNF do not affect operant sucrose self-administration. (A) Mean \pm SEM number of lever presses in 1 h after microinjection of GDNF into the SNc 10 min (0, 5, or 10 μ g per side) or 3 h (0 or 10 μ g per side) before the self-administration session. Two-way ANOVA with repeated measures showed significant main effects [lever: $F_{(1,32)} = 92.72, P < 0.001$; treatment: $F_{(4,32)} = 4.03, P < 0.01$] and a significant interaction between both factors [$F_{(4,32)} = 3.80, P < 0.02$]. Post hoc analysis showed significant differences in lever presses for ethanol between the 3-h GDNF pretreatment and the 10-min pretreatment ($P < 0.05$), but not with the PBS control ($P = 0.12$) ($n = 9$). (B) Mean \pm SEM number of lever presses in 1 h after GDNF microinjection into the VTA 10 min (0, 5, or 10 μ g per side) or 3 h (0 or 10 μ g per side) before sucrose self-administration. Two-way ANOVA with repeated measures showed a significant effect of lever [$F_{(1,20)} = 13.86, P < 0.01$] but no effect of treatment and no interaction [$F_{(4,20)} = 0.13$ and $F_{(4,20)} = 0.12$, respectively, nonsignificant] ($n = 8$).

of a naturally rewarding substance, sucrose. As shown in Fig. 2B, intra-VTA injections of GDNF did not affect lever-press responding for sucrose. Hence, the decrease in ethanol self-administration induced by intra-VTA infusion of GDNF was not due to a general attenuation of motivation or to a change in locomotor activity.

GDNF Activates the MAPK Extracellular Signal-regulated Kinase 1 and 2 (ERK1/2) in the VTA *in Vivo*, and Inhibition of ERK1/2 Activation Blocks GDNF-Induced Decreases in Ethanol Self-Administration. Next we set out to determine the signaling pathway that mediates the rapid actions of GDNF on ethanol consumption. The MAPK signaling pathway is one of the major downstream pathways activated by GDNF (1), and *ex vivo* studies suggest that GDNF rapidly modulates the activity of mesencephalic dopaminergic neurons via this intracellular pathway (31, 32). Therefore, we first assessed whether intra-VTA infusion of a behaviorally effective dose of GDNF (10 μ g) activates ERK1/2, a key enzyme in the MAPK signaling pathway. As shown in Fig. 3A, GDNF infusion induced a robust increase in ERK1/2 phosphorylation (i.e., activation) in the VTA, which was not observed in the control side infused with PBS. Importantly, a significant fraction of the phospho-ERK1/2 immunoreactivity was localized to tyrosine hydroxylase-positive neurons (Fig. 3), suggesting that activation of the GDNF pathway in the VTA leads to the activation of the MAPK pathway in dopaminergic neurons. Next, to examine the involvement of the MAPK signaling pathway in the attenuation of ethanol self-administration by GDNF, we blocked the activation of ERK1/2 in the VTA by inhibition of MAPK/ERK kinase (MEK), the upstream kinase that phosphorylates and activates ERK1/2 (33). As shown in Fig. 4A, intra-VTA infusion of the MEK inhibitor U0126 (34) prevented the decrease in ethanol self-administration induced by GDNF. However, intra-VTA infusion of the PI3K inhibitor wortmannin (34) did not alter the GDNF-mediated decrease in ethanol self-administration (Fig. 4B), suggesting that PI3K is not involved in GDNF's regulation of ethanol consumption. We could not assess whether PLC γ also contributes to GDNF's actions, as intra-VTA infusion of its inhibitor, U73122 (35), alone resulted in a reduction in ethanol self-administration (Fig. 4C) and

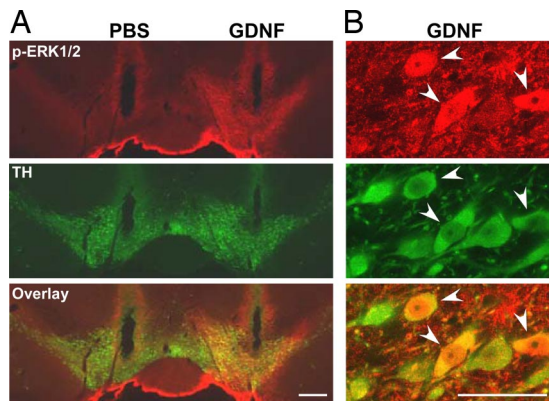


Fig. 3. GDNF activates ERK1/2 in midbrain dopaminergic neurons *in vivo*. Shown is dual-channel immunofluorescence for phospho-ERK1/2 (p-ERK1/2, red), tyrosine hydroxylase (TH, green), and overlay (yellow). (A) Images depict ERK1/2 phosphorylation in the midbrain 10 min after GDNF (right brain side) or PBS (left side) infusion into the VTA. Images are representative of results from three rats (nine sections per rat). (Scale bar: 500 μm .) (B) Enlarged image of VTA area infused with GDNF. The arrowheads point to cells immunostained both for p-ERK1/2 and tyrosine hydroxylase. (Scale bar: 50 μm .)

therefore might have masked the effect of GDNF. Together, these results suggest a crucial role for the MAPK signaling pathway in the decrease in ethanol self-administration observed after microinjection of GDNF into the VTA.

Intra-VTA Microinjection of GDNF Blocks Reacquisition of Ethanol Self-Administration. Relapse to alcohol use is one of the core features of alcoholism and is the main problem in the treatment of alcohol dependence (36, 37). Reacquisition, a rapid return of responding when the outcome is made available again after a period of extinction, is a measure of relapse (38, 39) that is especially relevant for therapies that seek to extinguish drug-related behaviors (40). We therefore tested whether intra-VTA infusion of GDNF would alter reacquisition of operant ethanol self-administration after a period of extinction. To obtain a one-session reacquisition, the retrieval of operant ethanol self-administration was triggered with a prime of a noncontingent delivery of ethanol (0.2 ml, 10%) in the reward port when the test session started, as described in *Materials and Methods*.

As shown in Fig. 5A, this procedure induced a rapid and effective reacquisition of ethanol self-administration. Importantly, intra-VTA infusion of GDNF 10 min before the beginning of the session blocked this reacquisition of operant responding

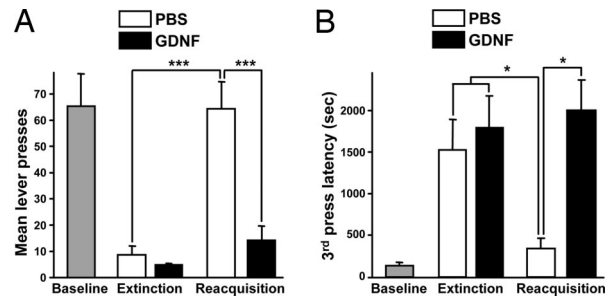


Fig. 5. Intra-VTA injection of GDNF blocks reacquisition of operant ethanol self-administration. (A) Mean \pm SEM number of lever presses in 1 h after GDNF microinjection into the VTA (0 or 10 $\mu\text{g}/1 \mu\text{l}$ per side) 10 min before the reacquisition test. Two-way ANOVA with repeated measures showed significant main effects [lever: $F_{(1,32)} = 40.52$, $P < 0.001$; treatment: $F_{(4,32)} = 15.31$, $P < 0.001$] and a significant interaction between both factors [$F_{(4,32)} = 20.97$, $P < 0.001$]. Baseline represents the mean responding for the last 4 days of self-administration training, and Extinction represents the mean lever presses during the final extinction session. (B) Mean \pm SEM latency in seconds to the third press (first reward) during the baseline, the final extinction session, and the reacquisition test for the PBS and GDNF conditions ($\chi^2 = 12.78$, $P < 0.01$). *, $P < 0.05$; ***, $P < 0.001$ ($n = 9$).

for ethanol as the active lever responding after GDNF treatment was not significantly different from active lever-press responding at the end of extinction ($P = 0.41$), or from inactive lever responding during the reacquisition session ($P = 0.20$). In addition, we observed a significant reduction of the latency to the third press, i.e., the first reward, relative to the latency on the last day of extinction in vehicle-treated rats (Fig. 5B and Table S1), indicating that this rapid reacquisition was promoted by the ethanol prime. This result also suggests that the ethanol prime reinstated interest in the reinforced lever. Interestingly, the ethanol priming effect was completely blocked by intra-VTA infusion of GDNF (Fig. 5B), as the latencies to the third press during the reacquisition test and on the last day of extinction were similar. This observation was further supported by the analysis of cumulative active lever-press responding (Fig. S3) showing a significant delay in the initiation of lever pressing for ethanol in GDNF-treated rats. Taken together, these data suggest that the ability of an ethanol prime to induce rapid reacquisition of operant ethanol self-administration is blocked by the application of GDNF.

Discussion

We identified a very rapid effect of GDNF to selectively reduce ethanol self-administration in two different paradigms; in one

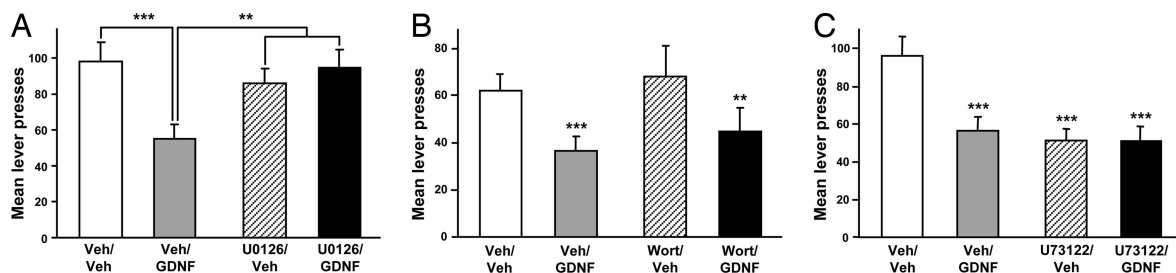


Fig. 4. Inhibition of the MAPK pathway blocks GDNF-induced decreases in ethanol self-administration. (A–C) Mean \pm SEM number of lever presses in 1 h after microinjection into the VTA of kinase inhibitors or the appropriate vehicles and GDNF (10 μg per side) or PBS, 1 h and 10 min before the self-administration session, respectively. (A) The MAPK inhibitor U0126 was injected at a concentration of 500 ng per side. Two-way ANOVA with repeated measures showed significant main effects [lever: $F_{(1,30)} = 249.52$, $P < 0.001$; treatment: $F_{(3,30)} = 3.03$, $P < 0.05$] and a significant interaction between both factors [$F_{(3,30)} = 6.19$, $P < 0.01$] ($n = 10$). (B) The PI3K inhibitor wortmannin was injected at a dose of 50 ng per side. Two-way ANOVA with repeated measures showed significant main effects [lever: $F_{(1,24)} = 35.61$, $P < 0.001$; treatment: $F_{(3,24)} = 6.06$, $P < 0.01$] and a significant interaction between both factors [$F_{(3,30)} = 4.89$, $P < 0.01$] ($n = 9$). (C) The PLC inhibitor U73122 was injected at a dose of 100 ng per side. Two-way ANOVA with repeated measures showed significant main effects [lever: $F_{(1,30)} = 95.06$, $P < 0.001$; treatment: $F_{(3,24)} = 12.51$, $P < 0.001$] and a significant interaction between both factors [$F_{(3,30)} = 13.05$, $P < 0.001$] ($n = 10$). **, $P < 0.01$; ***, $P < 0.001$.

paradigm, rats self-administer relatively low doses of ethanol whereas in the other rats self-administer relatively high levels of ethanol after a history of high consumption (Fig. S2). This action of GDNF is specifically mediated by the VTA, a primary site for the rewarding effects of ethanol (14), via the activation of the MAPK signaling pathway. We also demonstrate that GDNF infused into the VTA only 10 min before the operant test session completely blocked reacquisition of ethanol self-administration. Together, these findings indicate that GDNF in the VTA rapidly reduces ethanol drinking and relapse.

To our knowledge, this is the first evidence of a rapid action (within minutes) of a growth factor on drug consumption and relapse. As such, it is likely that GDNF's actions are mediated via a nontranscriptional mechanism. Several *ex vivo* studies suggest that GDNF acutely regulates the function of dopaminergic neurons in the midbrain. GDNF treatment of mesencephalic dopaminergic neurons increased the activity of tyrosine hydroxylase within minutes (32), as well as the excitability of the neurons by inhibition of A-type K^+ channels (31). GDNF was also shown to rapidly increase synaptic transmission by potentiation of Ca^{2+} channels (41). Interestingly, the rapid modulation of tyrosine hydroxylase and A-type K^+ channel activity depended on the activation of MAPK (31, 32). Here we show that *in vivo* administration of GDNF results in rapid activation of the MAPK signaling pathway in the VTA and that blocking the MAPK signaling pathway in this brain region prevents the modulation of ethanol self-administration by GDNF. Thus, it is plausible that the growth factor rapidly modifies neuronal excitability via activation of the MAPK pathway, and by doing so GDNF alters the incentive value and/or reinforcing strength of ethanol.

Activation of the MAPK signaling pathway, but not the PI3K pathway, within the VTA is critical for the rapid action of GDNF on ethanol self-administration. However, the role of the PLC γ /PKC could not be assessed because administration of the PLC inhibitor alone reduced ethanol self-administration, suggesting that PLC is involved in mechanisms that underlie consumption of ethanol. This result is not entirely surprising because the involvement of several PKC isozymes in ethanol-drinking behaviors has been documented (42).

We also show that the action of GDNF on ethanol self-administration is specific to the VTA, because infusion of effective doses of GDNF in the neighboring SNc dopaminergic area did not affect ethanol self-administration. This result is particularly striking because the immunocytochemistry experiment suggests that GDNF infused into the VTA can diffuse to the proximal part of the SNc and activate the MAPK signaling pathway in this structure as well. Therefore, these results strongly suggest that activation of the MAPK signaling pathway by GDNF specifically in the VTA reduces ethanol self-administration.

Interestingly, the ability of GDNF to reduce ethanol self-administration was observed 3 h after infusion. As a growth factor, it is possible that GDNF induces several transcriptional changes that could sustain its action. For example, it has been shown that GDNF increases the expression of tyrosine hydroxylase and the dopamine transporter (43), inducing modifications of the mesolimbic system that persist beyond the activation and termination of GDNF signaling. Another possible mechanism is a sustained effect by GDNF itself, as we recently showed that GDNF up-regulates its own expression, leading to a sustained activation of the GDNF signaling pathway (27).

Intra-VTA infusion of GDNF did not alter self-administration of sucrose, a natural reward, suggesting that the reduction in lever-press responding for ethanol was not due to nonspecific motor effects of GDNF. Other pharmacological manipulations have been previously reported to modulate the seeking and consumption of drugs of abuse and ethanol but not natural rewards (26, 44–46). Although it has been widely accepted that

drugs of abuse and natural rewards have the mesolimbic dopaminergic system as a common substrate, several studies have suggested that processes involved in the rewarding effects of sucrose and ethanol are different. For example, both sucrose self-administration and ethanol self-administration induce an increase in dopamine concentration in the nucleus accumbens (47–49); however, in the case of sucrose, this seems to be linked to locomotor or operant/learning processes because neither the first ingestion of sucrose nor the unpredicted delivery of sucrose during operant self-administration changes dopamine levels in the nucleus accumbens, whereas ethanol does (50, 51). Also, microinjection of a D2 agonist in the anterior part of the VTA decreases ethanol but not saccharin self-administration (52), and microinjection of D1/2 agonists/antagonists produces distinct effects on cocaine and sucrose self-administration (53). Together, these results suggest a specific involvement of GDNF in the VTA in ethanol and/or addictive processes but not in general rewarding and/or motivational mechanisms.

Relapse is one of the main challenges in the treatment of alcohol abuse (36, 37). A therapeutic approach to treat relapse is to prevent the initial lapse, or the consequence of the initial lapse, that spirals into relapse (54–56). In this regard, rapid reacquisition of an operant response for a drug of abuse after re-exposure to the drug is a particularly relevant relapse model, especially in the case of therapies that seek to extinguish drug-taking behaviors (39, 40). We found that re-exposure to the exteroceptive properties of ethanol (i.e., the sensory cues of the ethanol prime, such as the taste and the specific odor) induced a rapid and effective reacquisition of ethanol self-administration. Importantly, intra-VTA infusion of GDNF 10 min before the operant session blocked this reacquisition of responding for ethanol. This effect of GDNF is likely mediated by a specific action on some reacquisition mechanism(s), because the ethanol prime completely lost its ability to rapidly reinstate interest in the reinforced lever and responding for ethanol. This is illustrated by a representative example of one individual rat's pattern of responding during the reacquisition test (Fig. S3B), showing a significant delay to perform the first ratio in the GDNF condition. This effect of the ethanol prime suggests that GDNF reduces not only ethanol consumption but also ethanol seeking.

In conclusion, our results suggest a unique fast-acting effect of GDNF mediated by the MAPK pathway in the VTA that selectively reduces ethanol consumption. Moreover, GDNF in the VTA blocked reacquisition of operant ethanol self-administration (a model of relapse), suggesting that GDNF is involved in different aspects of ethanol-drinking and -seeking behaviors. Our results also put forward the potential use of targets within the GDNF pathway for the development of treatment against alcohol abuse and, most importantly, relapse.

Materials and Methods

Reagents. Reagents are detailed in *SI Materials and Methods*.

Animals. Male Long-Evans rats (Harlan; 350–400 g at the time of surgery) were housed under a 12-h light/dark cycle (lights on at 0700 hours) with food and water available ad libitum. All animal procedures in this report 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.

Operant Ethanol Self-Administration. Rats were habituated to drinking ethanol in their home cages by exposure to 10% ethanol in tap water (vol/vol) mixed with a decreasing concentration of sucrose (10%, 5%, and 0%, wt/vol). After 3 weeks, operant ethanol self-administration training commenced. The self-administration chambers contain two levers: an active (ethanol) lever, for which presses result in delivery of 0.1 ml of a 10% ethanol solution, and an inactive lever, for which responses are counted as a measure of nonspecific behavioral activity but no programmed events occur. After two to three nights in the chambers to allow acquisition of a lever-press response for a solution of

10% ethanol under a fixed ratio 1 (FR1; one press delivers one reward), 60-min sessions were conducted 5 days per week, with the schedule requirement increased to FR3 over the first week. Because the level of presses on the inactive lever was low after acquisition of the self-administration paradigm (<10 presses), and the activity on this lever was not affected by any of the experimental treatments, this measure was removed from the figures for better clarity but taken into account in the statistical analyses. After 2 months of training, surgery to implant cannulae was conducted.

Operant Self-Administration After Intermittent Access to Ethanol. Intermittent access to 20% ethanol in tap water (vol/vol) was provided according to previous studies (29, 45). After rats achieved a stable baseline of consumption, they were trained to self-administer ethanol in operant chambers as described above, with the reinforcer being 0.1 ml of a 20% ethanol solution and the session length shortened to 30 min after 2 weeks of training. Surgery to implant cannulae was conducted after 2 weeks of stable responding in 30-min sessions.

Sucrose Self-Administration. Rats were initially trained under FR1 by using 8% sucrose (wt/vol) as the reinforcer during two overnight sessions. The FR schedule was then progressively increased to FR3, and sucrose concentration was progressively decreased to 2%. The rats were trained under this final schedule 5 days per week in 60-min sessions. As for the ethanol experiments, the inactive lever measure was removed from the figures but not from the statistical analyses. Because the training takes only 3 weeks, surgery to implant the cannulae was conducted before the behavioral procedure.

Surgery and Microinjection. Bilateral guide cannulae (C235G-2.0, 26 gauge; Plastics One) were aimed dorsal to the VTA (5.6 mm posterior to bregma, 1.0 mm mediolateral, 8.0 mm ventral to the skull surface) or the SNc (5.4 mm posterior to bregma, 2.6 mm mediolateral, 6.8 mm ventral to the skull surface), according to Paxinos and Watson (57). Drug or vehicle was infused into the VTA or the SNc of gently restrained rats via injection cannulae extending 0.5 mm beyond the guide cannula tip. All subjects received each treatment in a counterbalanced manner, with one injection per week (see *SI Materials and Methods* for details).

Intra-VTA Microinjection of MAPK, PI3K, and PLC Inhibitors. A total of 0.5 μ l of U0126 (1 μ g/ μ l), wortmannin (0.1 μ g/ μ l), U73122 (0.2 μ g/ μ l), or the appropri-

ate vehicle per side was infused into the VTA 1 h before the intra-VTA infusion of GDNF (0.5 μ l of a 10 μ g/ μ l solution per side) (see *SI Materials and Methods* for details).

Immunohistochemistry. GDNF or PBS was infused in the VTA 10 min before perfusion and removal of the brain as described in *SI Materials and Methods*. Perfusion and immunocytochemistry procedures are as described in ref. 58 and in *SI Materials and Methods*.

Intra-VTA Microinjection of GDNF and Reacquisition of Ethanol Self-Administration. After 2 months of ethanol self-administration training, rats underwent daily 60-min extinction sessions (no reward after active lever responses). After 14 days of extinction, half of the rats were infused with 10 μ g/ μ l of GDNF per side, and the other half were injected with PBS into the VTA 10 min before the reacquisition test session, in which a 0.2-ml drop of 10% ethanol was delivered into the reward port noncontingently to the lever response when the session started (the ethanol prime). Subsequently, three lever presses on the active lever resulted in the delivery of 0.1 ml of the reinforcer, as during the self-administration procedure. After 2 weeks of reacquisition of ethanol self-administration followed by nine further extinction sessions, a second reinstatement test session was conducted with the drug treatment reversed.

Histology. Locations of cannulae were verified in 60- μ m coronal sections stained with thionin. Only data from subjects with injectors located in the region of interest (Fig. S4) were included in the analysis.

Statistical Analyses. Each experiment was conducted in a within-subjects design. The number of lever presses was analyzed by using ANOVAs with repeated measures. Significant main effects and interactions of the ANOVAs were further investigated by using the Student–Newman–Keuls test. Because the data did not conform to a normal distribution, the latency to the first reward was analyzed with the Friedman repeated-measures ANOVA on ranks. Significant effects of these ANOVAs were further investigated by using the nonparametric variant of the Student–Newman–Keuls test (Sigstat 2004; Systat).

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