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Excessive alcohol consumption is blocked by glial cell line–derived neurotrophic factor

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Abstract

We previously found that activation of the glial cell line–derived neurotrophic factor (GDNF) pathway in the ventral tegmental area (VTA) reduces moderate alcohol (ethanol) intake in a rat operant self-administration paradigm. Here, we set out to assess the effect of GDNF in the VTA on excessive voluntary consumption of ethanol. Long–Evans rats were trained to drink large quantities of a 20% ethanol solution in an intermittent-access two-bottle choice drinking paradigm. The rats were given three 24-h sessions per week, and GDNF's actions were measured when rats achieved a baseline of ethanol consumption of 5.5 g/kg/24 h. We found that microinjection of GDNF into the VTA 10 min before the beginning of an ethanol-drinking session significantly reduced ethanol intake and preference, but did not affect total fluid intake. We further show that GDNF greatly decreased both the first bout of excessive ethanol intake at the beginning of the session, and the later consummatory activity occurring during the dark cycle. These data suggest that GDNF is a rapid and long-lasting inhibitor of "binge-like" ethanol consumption.

Keywords

Alcohol; GDNF; VTA; Addiction; Ethanol; Growth factor

Introduction

Glial cell line–derived neurotrophic factor (GDNF) is a secreted protein that was initially identified in a glial-derived cell line (Lin et al., 1993). GDNF is an essential growth factor for the development of kidneys and spinal cord motoneurons (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), and plays a critical role in promoting the survival, regeneration, and maintenance of the mature phenotype of distinct central and peripheral neuronal populations, including the midbrain dopaminergic neurons (Beck et al., 1995; Granholm et al., 2000; Kowsky et al., 2007; Lin et al., 1993; Pascual et al., 2008; Tomac et al., 1995a). In the mesolimbic system, GDNF is produced in striatal neurons (Barroso-Chinea et al., 2005; Pochon et al., 1997) and is retrogradely transported by dopaminergic neurons of the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) in the adult brain (Ai et al., 2003; Barroso-Chinea et al., 2005; Kordower et al., 2000; Lapchak et al., 1997; Tomac et al., 1995b), where the GDNF receptors, GFRα1 and Ret, are highly expressed (Burazin and Gundlach, 1999; Glazner et al., 1998; Golden et al., 1998; Matsuo et al., 2000; Trupp et al., 1997). Ligation of GDNF to GFRα1 leads to the recruitment and activation of receptor tyrosine kinase Ret, and to the consequent activation of the mitogen-activated protein kinase (MAPK),

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phosphoinositide 3-kinase (PI3K) and phospholipase Cγ (PLCγ) pathways (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003).

In recent years, the role for GDNF as a negative regulator of biochemical and behavioral adaptations to drugs of abuse has been reported (Ron and Janak, 2005). Specifically, Messer et al. (2000) showed that infusion of GDNF into the VTA reversed biochemical adaptations to long-term exposure to cocaine and morphine, and blocked cocaine conditioned place preference (CPP). Transplantation of cells engineered to overexpress GDNF or delivery of nanoparticles conjugated to GDNF into the striatum were shown to reduce acquisition of rat cocaine self-administration (Green-Sadan et al., 2003, 2005), and increasing GDNF expression in the brain reduced the sensitivity of mice to behavioral responses to methamphetamine (Niwa et al., 2007b). In addition, heterozygous GDNF knockout (GDNF HET) mice exhibited exaggerated behaviors related to morphine, cocaine, and methamphetamine exposure compared to their wild-type (GDNF WT) littermates (Airavaara et al., 2007; Messer et al., 2000; Niwa et al., 2007a, b; Yan et al., 2007). For example, CPP to psychos-timulants, as well as motivation to self-administer and seek methamphetamine are potentiated in GDNF HET mice compared to GDNF WT mice (Messer et al., 2000; Niwa et al., 2007b; Yan et al., 2007).

During the past several years, we obtained evidence suggesting that GDNF negatively regulates behaviors associated with exposure to ethanol. We found that systemic administration of the naturally occurring alkaloid, ibogaine, to rats increases the expression of GDNF in the VTA, leading to the reduction of rat operant self-administration of ethanol (He et al., 2005). We further showed that GDNF reverses a biochemical adaptation resulting from prolonged exposure of cells to ethanol. Specifically, we found that treatment of the dopaminergic-like SH-SY5Y cell line with ethanol leads to an increase in the protein stability of tyrosine hydroxylase, which was reversed by GDNF (He and Ron, 2008). Importantly, we found that a single infusion of GDNF into the VTA leads to a rapid and sustained reduction of rat operant ethanol self-administration (Carnicella et al., 2008). Interestingly, GDNF did not affect operant sucrose self-administration, suggesting a selective effect of GDNF in the VTA on ethanol-mediated actions. We also found that the actions of GDNF to attenuate the motivation to consume ethanol are mediated via the activation of the MAPK pathway (Carnicella et al., 2008). Finally, we showed that a single infusion of GDNF into the VTA blocks reacquisition of ethanol self-administration after a period of extinction (Carnicella et al., 2008).

Our previous study showed that GDNF is a potent inhibitor of the motivation of rats to consume ethanol; however, the level of ethanol intake in the operant self-administration paradigm was moderate (Carnicella et al., 2008). Therefore, we were interested in determining the effect of GDNF in the VTA on excessive ethanol consumption using a model that resembles “binge-drinking” in humans. To do so, we used the intermittent-access 20% ethanol two-bottle-choice drinking paradigm, in which repeated cycles of deprivation result in the voluntary consumption of large amounts of ethanol in a short period of time (Carnicella et al., 2008; Simms et al., 2008; Wise, 1973).

Materials and methods

Reagents

GDNF was purchased from R&D systems (Minneapolis, MN). β-Nicotinamide adenine dinucleotide (NAD) and alcohol dehydrogenase (ADH) were purchased from Sigma (St. Louis, MO).

Animals

Male Long–Evans rats (Harlan; 400–450 g at the time of the surgery) were housed under a 12-h light/dark cycle (lights on at 7:00 a.m.) with food and water available ad libitum, and kept in conditions of constant temperature (23°C) and humidity (50%). 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.

Intermittent-access 20% ethanol two-bottle-choice drinking paradigm

Intermittent-access of 20% ethanol was similar to the paradigm described previously by Simms et al. (2008) and Stuber et al. (2008). Specifically, animals were given 24-h concurrent access to one bottle of 20% vol/vol ethanol in tap water and one bottle of water, starting at 11:00 a.m. on Monday, Wednesday, and Friday, with 24 or 48-h ethanol-deprivation periods in between the ethanol-drinking sessions. The placement (left or right) of each solution was alternated between each session to control for side preference. The water and ethanol bottles were weighted after 30 min, 4 h, and 24 h of access, according to the experimental protocol. A bottle containing water in a cage without rats was used to evaluate the spillage due to the experimental manipulations during the test sessions. The spillage was always ≤0.5 mL (<2.5% of the total fluid intake). Blood ethanol concentration (BEC) determination, surgery, and microinjection procedures started after 21 ethanol access sessions, when rats maintained a stable baseline of ethanol consumption.

Blood ethanol concentration measurements

Thirty minutes after the re-introduction of the ethanol bottle, rats were briefly anesthetized with isoflurane and blood was collected from the lateral tail vein with heparinized capillary tubes. Serum was extracted with 3.4% trichloroacetic acid, centrifuged for 5 min at 2,000 rpm, and then assayed for ethanol content using the NAD-NADH enzyme spectrophotometric method (Weiss et al., 1993; Zapata et al., 2006). BECs were determined using a standard calibration curve.

Surgery and microinjection

Rats were anesthetized continuously with isoflurane (Baxter Health Care Corporation, Deerfield, IL). Bilateral guide cannulae (C235G-1.5, 26 ga, Plastics One, Roanoke, VA) were aimed dorsal to the VTA (5.6 mm posterior to bregma, 0.75 mm mediolateral, 8.0 mm ventral to the skull surface), according to Paxinos and Watson (1998). After 3 days of recovery, rats returned to the intermittent-access 20% ethanol procedure and microinjections began after three ethanol-drinking sessions, when drinking returned to a stable baseline. GDNF (10 μg/μL) or vehicle phosphate-buffered saline (PBS) were microinjected 10 min before the beginning of a 24-h ethanol-access session. A total of 1 μL of GDNF or vehicle was infused over 2 min into the VTA of gently restrained rats via injection cannulae extending 0.5 mm beyond the guide cannula tip. Injection cannulae were left in place for an additional 2 min. The GDNF microinjection experiment was conducted within 1 week. On Monday, half of the animals were microinjected with GDNF and the other half with vehicle, 10 min prior to the beginning of the 24-h ethanol-drinking session. The second ethanol-access session that started on Wednesday was conducted without any treatment, allowing ethanol intake to return to baseline level (vehicle- and GDNF-treated animals showed similar levels of basal level of ethanol consumption throughout the experiment; data not shown). Ten minutes prior to the third ethanol-access session of the week (on Friday), animals were microinjected with GDNF or vehicle, but with the drug treatment reversed. The parameters used in this study (i.e., site and time of injection as well as the GDNF concentration) were previously found to effectively reduce operant ethanol self-administration (Carnicella et al., 2008).

Histology

Locations of cannulae were verified in 60-μm coronal sections of paraformaldehyde-fixed tissue stained with thionin.

Statistical analysis

The correlation between BEC (mg%) and ethanol intake (g/kg/30 min) was analyzed by linear regression. Experiments were conducted in a within-subjects design and analyzed by paired *t*-test or one-way analysis of variance with repeated measures. Preference was calculated as the percentage of ethanol solution consumed relative to total fluid intake (ethanol + water).

Results

Intermittent-access of Long–Evans rats to 20% ethanol results in heavy drinking and high concentration of ethanol in the blood

We used the intermittent-access to 20%ethanol paradigm to assess GDNF's actions on voluntary excessive consumption of ethanol. As previously reported (Carnicella et al., 2008; Simms et al., 2008), rats showed a significant escalation of ethanol intake [$F(20, 440) = 24.05$, $P < .001$] and preference [$F(20, 440) = 28.49$, $P < .001$] across sessions of access to 20% ethanol (Fig. 1A, B) and reached a stable baseline of ethanol consumption (5.39 ± 0.37 g/kg/24 h) and preference (49.5 ± 4.10%) after 15 sessions. Interestingly, we observed that at the beginning of the session, rats consumed large quantities of ethanol very rapidly and the average ethanol intake was 1.39 ± 0.11 g/kg in the first 30 min of the session, which corresponded to ~25% of the total ethanol consumed within 24 h (Fig. 1C). BEC was determined immediately after the 30-min period of access to ethanol, and the amount of ethanol consumed significantly correlated with the measured BEC ($r^2 = 0.63$, $P < .001$, Fig. 1D). The BEC ranged from 7.1 to 158.6 mg% (1.5–34 mM) with an average of 80.9 ± 7.2 mg% (17.5 ± 1.5 mM), and in about 50% of the rats the BEC was above average (Fig. 1D). Approximately 60% of the total ethanol intake (3.28 ± 0.31 g/kg) was consumed during the second period (4–24 h) (Fig. 1C), and while measuring ethanol intake over the 12-h period of the dark cycle on a subset of animals, we observed that the majority of ethanol was consumed during this time (3.65 g/kg ethanol during the dark cycle out of a 4.65 g/kg intake during the 4–24 h period).

Intra-VTA microinjection of GDNF reduces excessive voluntary ethanol intake

Next, we assessed the effect of intra-VTA administration of GDNF on excessive voluntary ethanol intake. Fig. 2 displays the placement of the injector tips of the subjects included in the behavioral analysis. The injection sites were confined within the posterior region of the VTA. Rats were infused with a dose of 10 μg/side of GDNF because it was the most effective dose to reduce operant ethanol self-administration (Carnicella et al., 2008).

As shown in Fig. 3A, intra-VTA microinjection of GDNF 10 min before the beginning of the session significantly decreased ethanol intake over the 24-h access period [$T(13) = 2.80$, $P < .02$]. Infusion of GDNF into the VTA also reduced the preference for ethanol [$T(13) = 2.46$, $P < .05$; Fig. 3B]. Total fluid intake was unchanged [$T(13) = 0.76$, $P = .48$; Fig. 3C], and the decrease in ethanol intake was accompanied by an increase in water intake [$T(13) = 2.66$, $P < .05$; Fig. 3D]. Importantly, we observed that GDNF application into the VTA 10 min before the beginning of the session prevented the "binge-like" drinking behavior observed during the first 30 min of drinking [$T(13) = 8.49$, $P < .001$; Fig. 4A]. In addition, GDNF was effective in reducing ethanol intake during the second period of consumption [$T(13) = 2.19$, $P < .05$; Fig. 4B]. Together, these data suggest a rapid and sustained inhibitory effect of GDNF on excessive ethanol consumption over the 24-h ethanol access session.

Discussion

Here, we report that microinjection of GDNF into the VTA of rats significantly reduces excessive voluntary ethanol consumption in an intermittent-access 20% ethanol two-bottle-choice drinking paradigm. Importantly, we found that this inhibitory action of the growth factor was rapid and long lasting, as GDNF infused 10 min before the session decreased both the first bout of excessive ethanol intake and the later consummatory activity that occurred mainly during the dark cycle.

We observed that repetition of the 24- or 48-h ethanol deprivation periods induced episodes of heavy drinking for short periods of time that is reminiscent of binge-drinking in humans. Interestingly, binge drinking is defined by the National Institute Abuse and Alcoholism of (NIAAA) as a pattern of drinking that results in BEC to 80 mg% or above (NIAAA, 2004), which is similar to the average of the BEC we measured in the rats. This suggests that the majority of the animals in the intermittent-access 20% ethanol two-bottle choice drinking paradigm are highly motivated to consume ethanol for its pharmacological effects and as such, exhibit “binge-like” drinking behavior. Interestingly, the escalation in ethanol intake is not observed when rats are subjected to a continuous ethanol access (Wise, 1973 and data not shown), supporting a critical role of the repeated cycles of ethanol deprivation and consumption that result in neuroadaptative changes that lead to further increase in the ethanol intake. In line with this possibility, chronic intermittent, but not continuous, exposure to ethanol was found to significantly increase dopamine transporter levels within the nucleus accumbens (Healey et al., 2008), and voluntary chronic ethanol intake in the same intermittent-access 20% ethanol two-bottle choice drinking paradigm enhances postsynaptic AMPA receptor function in VTA neurons (Stuber et al., 2008).

Importantly, we found that GDNF acts as a potent inhibitor of excessive ethanol drinking. It is unlikely that this action of GDNF was due to nonspecific locomotor effects or a general decrease in motivation as the total fluid intake was unaffected by GDNF, and we previously showed that intra-VTA microinjection of GDNF did not alter operant self-administration of sucrose (Carnicella et al., 2008). Together, our data suggest that GDNF in the VTA acts specifically on ethanol-related behaviors.

Intra-VTA administration of GDNF resulted in a 70% and 50% decrease in ethanol intake during 30 min and 24 h access to ethanol, respectively. Previously, we found that infusion of GDNF into the VTA reduced operant self-administration of a 20% ethanol solution to a lesser extent (45–50%) (Carnicella et al., 2008). Although we cannot quantitatively compare the two paradigms, the results may suggest that GDNF may reduce free-choice drinking to a greater degree compared to a paradigm in which the animal has to work for alcohol as a reinforcer.

Due to the limited solubility of the growth factor, 1 μL of the solution was infused into the VTA. This volume is relatively high for the VTA, therefore it might be possible that the reduction of ethanol intake by GDNF was due to the diffusion of the growth factor to the neighboring dopaminergic area, the SNc. However, this is unlikely because our previous study shows that GDNF infusion into the SNc did not affect operant ethanol self-administration (Carnicella et al., 2008). GDNF can undergo anterograde and retrograde axonal transport (Ai et al., 2003; Kordower et al., 2000) and therefore, we cannot exclude the possibility that the effect of GDNF on ethanol intake is mediated by the growth factor’s actions in other brain areas. However, this is also unlikely for several reasons. First, VTA is a preferential site of action of GDNF as the GDNF receptors Ret and GFRα1 are highly expressed in this area in the adult brain (Burazin and Gundlach, 1999; Glazner et al., 1998; Trupp et al., 1997). In contrast, very low or negligible levels of GFRα1 and Ret are detected in the nucleus accumbens (Burazin and Gundlach, 1999; Glazner et al., 1998; Trupp et al., 1997). Second, the effect of

intra-VTA infusion of GDNF on ethanol intake is very rapid, within 10 min (present study and Carnicella et al., 2008), and we found that this period of time allowed GDNF to activate the MAPK pathway that is downstream to Ret in the VTA (Carnicella et al., 2008), but not in the brain areas targeted by VTA dopaminergic neurons, such as the nucleus accumbens or the prefrontal cortex (unpublished data, V. Kharazia, S. Carnicella, and D. Ron). Taken together, these data suggest that the effects of GDNF on ethanol-drinking behaviors are specifically mediated by the VTA. Nevertheless, it might be of great interest for future investigation to test whether GDNF can rapidly modulate ethanol-drinking behaviors when microinjected in other brain limbic regions expressing a high level of GDNF receptors, such as the amygdala or the habenula (Trupp et al., 1997).

GDNF, within minutes, dramatically reduced the bout of excessive ethanol intake that occurred at the beginning of the session. We previously showed that infusion of GDNF into the VTA very rapidly decreased operant ethanol self-administration and relapse (Carnicella et al., 2008). However, animals in the operant self-administration paradigm consumed relatively moderated amounts of ethanol (0.4–0.8 g/kg in 30 min or 1 h). The mechanisms underlying moderate and excessive consumption of ethanol are believed to be different (e.g., Koob, 2003), and as such, pharmacological substances can affect moderate and excessive drinking differently. For example, corticotropin-releasing factor 1 antagonists were shown to reduce only excessive ethanol self-administration in dependent rats (Funk et al., 2007), and acamprosate and naltrexone were found to be more efficient to decrease ethanol consumption in rats consuming high levels of ethanol (Simms et al., 2008). Interestingly, GDNF reduces both moderate levels of ethanol consumption (that resembles social drinkers in humans) and excessive ethanol consumption, a hallmark of alcoholism, suggesting that the growth factor inhibits a pathway common to adaptations that result in moderate and excessive ethanol intake.

The mechanism by which GDNF decreases ethanol-drinking behaviors is unknown. However, GDNF was reported to rapidly increase the excitability and function of mesencephalic neurons in vitro (Kobori et al., 2004; Wang et al., 2003; Yang et al., 2001), and acute injection of GDNF into the midbrain enhances dopaminergic transmission (Hebert et al., 1996; Hudson et al., 1995). The mesolimbic dopaminergic system is believed to play an important role in the reinforcing effects of ethanol and in the development of alcohol addiction (Gonzales et al., 2004; Weiss and Porrino, 2002). For example, ethanol can directly excite the dopaminergic neurons of the VTA (Brodie et al., 1999) and increase DA levels in the nucleus accumbens (Gonzales et al., 2004). In addition, pharmacological manipulations of the dopaminergic transmission within the mesolimbic system alter ethanol self-administration (Gonzales et al., 2004). Chronic exposure to high levels of ethanol results in substantial modifications of VTA dopaminergic neurons activity (reviewed in Diana et al., 2003) and a decrease in DA levels in the nucleus accumbens (Darden and Hunt, 1977; Weiss et al., 1996) during withdrawal. This DA hypofunction has been suggested to be a part of an allostatic process that leads to compulsive ethanol intake, prolongation of craving, and propensity to relapse to compensate for these DA deficits and the negative emotional state (e.g., dysphoric symptoms) associated with them (Fadda and Rossetti, 1998; Koob and Le Moal, 2001; Weiss et al., 1996; Weiss and Porrino, 2002). Therefore, it might be plausible that GDNF reduces ethanol consumption by adjusting the activity of VTA dopaminergic neurons and as a consequence, the accumbal DA levels.

We also observed that GDNF infusion into the VTA decreased the ethanol drinking that occurred over 20 h, mainly during the dark cycle, suggesting a long-lasting effect of the protein. As a growth factor, GDNF induces several transcriptional modifications (Airaksinen and Saarma, 2002). We previously showed that GDNF activates a positive feedback loop in which the growth factor increases its own expression, leading to a sustained activation of the GDNF signaling pathway (He and Ron, 2006). Therefore, upregulation of the *GDNF* gene could

account for its long-lasting action to reduce voluntary ethanol intake. However, as a fraction of exogenous GDNF was reported to be detected in the brain 24 h after the infusion (Lapchak et al., 1997; Tomac et al., 1995b), we cannot exclude the possibility that the long-lasting effect we observed is due to the presence of a portion of the exogenous GDNF throughout session.

Importantly, GDNF was highly effective in reducing heavy drinking after a short period of deprivation. These results are in line with our previous studies showing that GDNF in the VTA reduces reacquisition to lever presses for ethanol after a 2-week period of extinction (Carnicella et al., 2008). Curiously, we found that GDNF in the VTA was much more effective in inhibiting reacquisition of ethanol self-administration after the extinction period, than in reducing ethanol self-administration before the period of abstinence (Carnicella et al., 2008). Taken together, these data suggest an important protective role of GDNF in abstinence and relapse processes.

In summary, we have shown that GDNF in the VTA selectively reduces high levels of consumption and “binge-like” ethanol drinking that followed a short period of deprivation. Binge-drinking is becoming more prevalent during school and college years in North America and Europe, and is a strong predictor of future alcohol-related problems (e.g., Bloomfield et al., 2003). Moreover, heavy drinking is a factor that largely contributes to the onset and development of several major chronic diseases and alcohol use disorders (Lancaster, 1994; Rehm et al., 2003). Furthermore, lapse and relapse to alcohol drinking, which in many cases results in binge-drinking (Rösner et al., 2008), are fundamental issues in alcoholism treatment. The estimated rate of relapse is in the range of 70–80% within one year post-treatment (Dawson et al., 2007), and evidence suggests that approximately 90% of alcoholics are likely to experience at least one relapse episode over a 4-year period following treatment (NIAAA, 1989). Our data support the possibility that upregulation of GDNF expression and/or activation of the GDNF pathway may be valuable strategies for the development of treatment of excessive alcohol consumption and the prevention of relapse.

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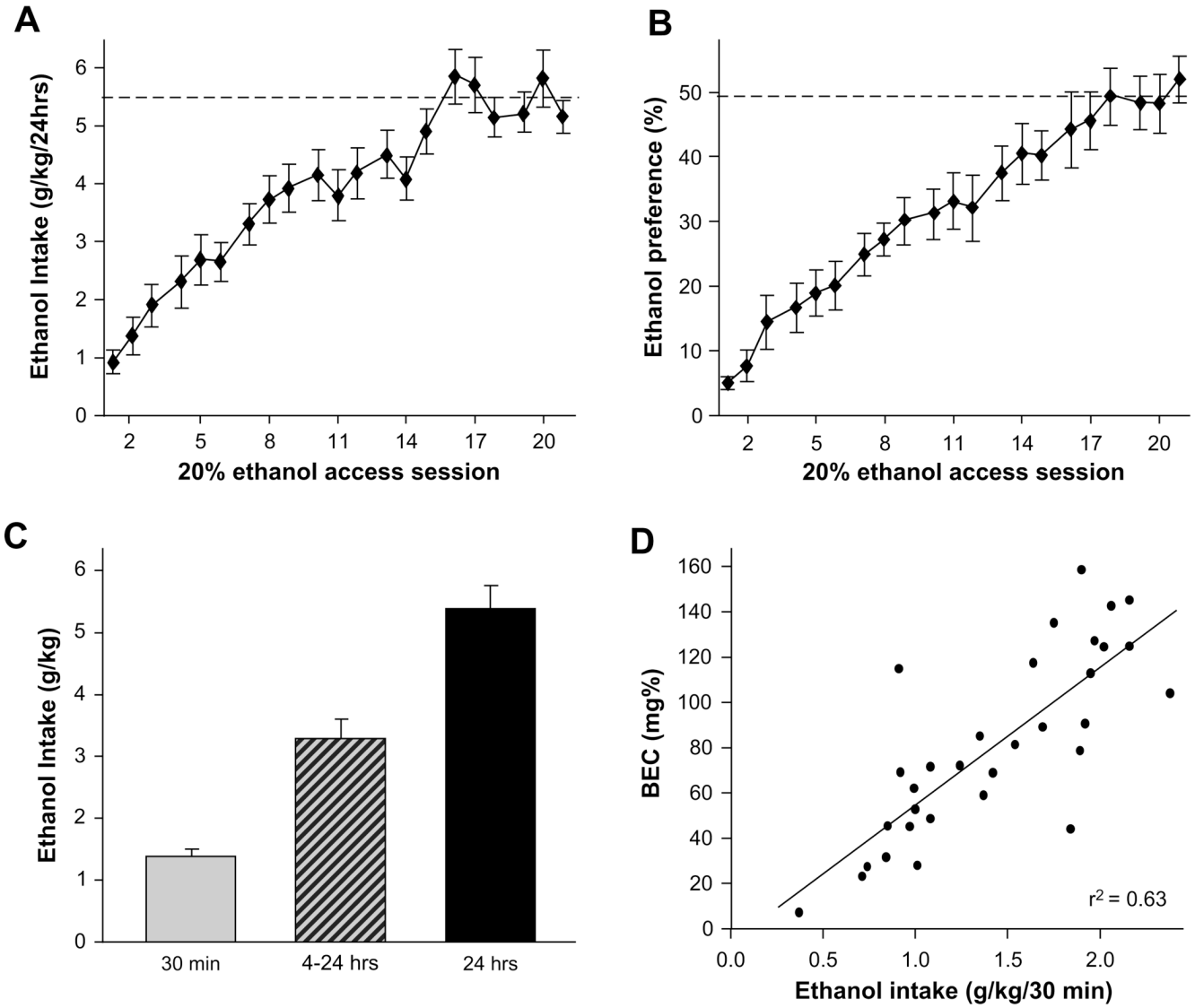

Fig. 1.

High level of ethanol consumption is obtained in an intermittent access two-bottle-choice drinking paradigm. Mean ± standard error of the mean (S.E.M.) of ethanol intake (A) and preference (B) during acquisition of voluntary ethanol consumption of a 20% ethanol solution, $n = 24$. (C) Mean ± S.E.M. of ethanol intake of the last three drinking sessions, during the first 30 min, the last 20 h (that includes the dark period of the cycle) and over the 24 h of the session, $n = 24$. (D) Correlation between the ethanol consumed during the first 30 min of the drinking session and the blood ethanol concentration (BEC) ($r^2 = 0.63$, $P < .001$). The average ethanol intake was 1.41 ± 0.12 g/kg/30 min, ranging from 0.37 to 2.38 g/kg. The BEC measurements include the 24 animals used in the present study and eight animals from an earlier work (Carnicella et al., 2008), $n = 32$.

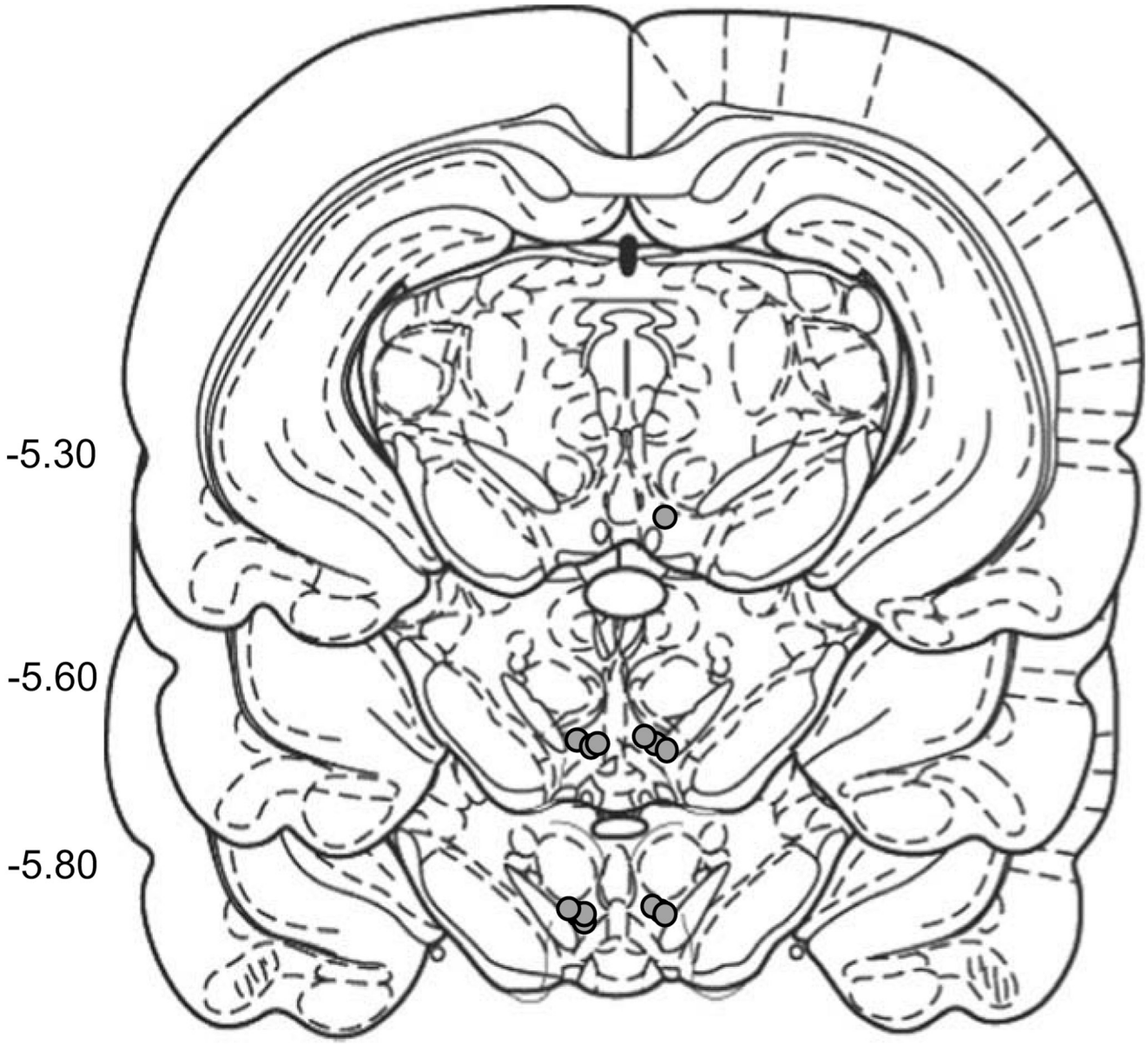

Fig. 2.

Schematic representation of the injection cannulae placements in coronal sections (Paxinos and Watson, 1998). The location of the injector tips is represented by *gray circles*. Numbers indicate the distance posterior to bregma in millimeters. One rat was discarded due to cannulae misplacement.

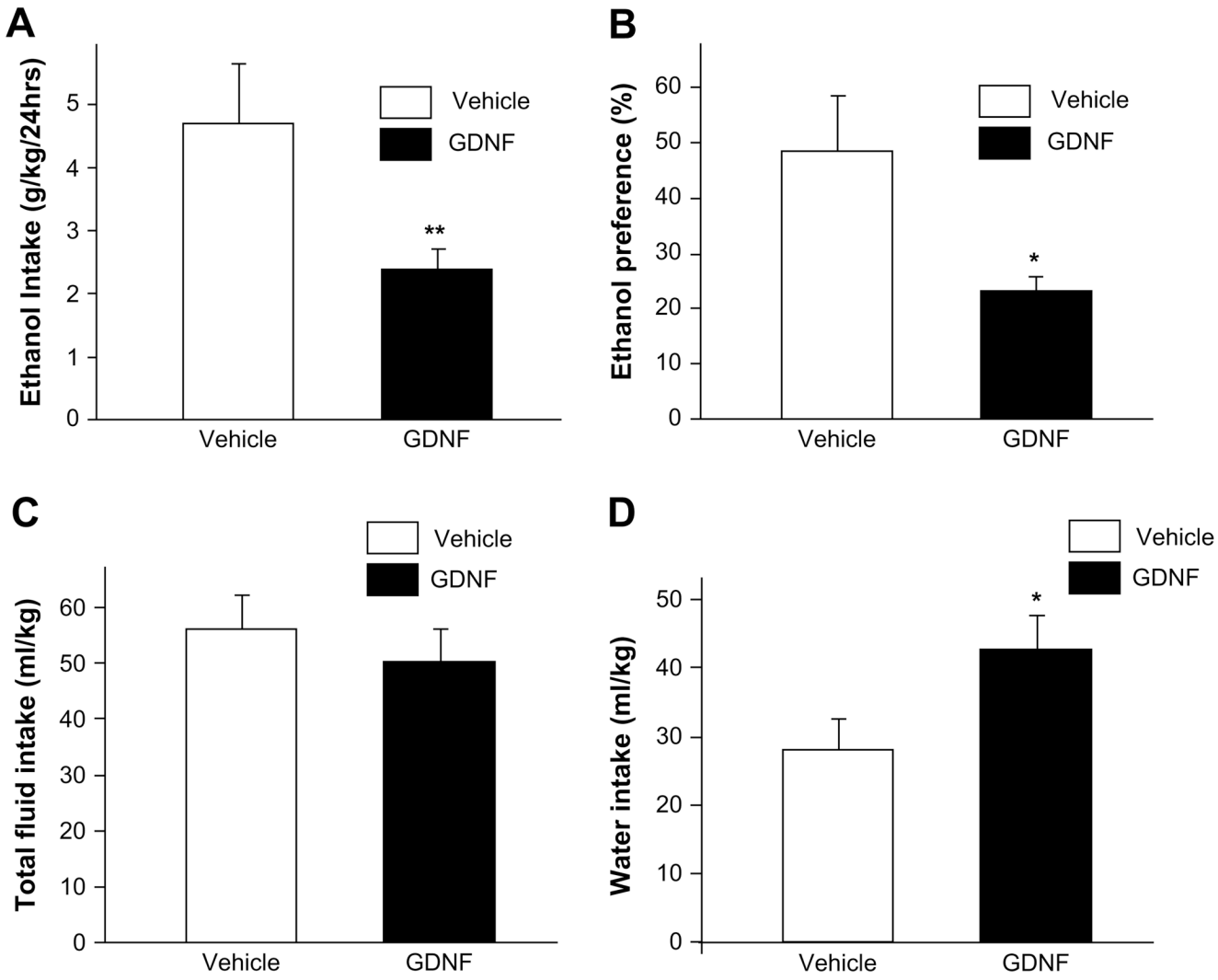

Fig. 3.

Intraventral tegmental area microinjection of glial cell line–derived neurotrophic factor (GDNF) reduces high levels of voluntary ethanol consumption in rats. GDNF (10 μg/side) or vehicle was microinjected into the ventral tegmental area 10 min before a 24-h ethanol-drinking session in rats trained to consume a 20% solution of ethanol. Mean ± standard error of the mean of ethanol intake (A), ethanol preference (B), total fluid intake (C), and water intake (D) during the 24-h session, $n = 7$; *$P < .05$; **$P < .02$ compared to vehicle.

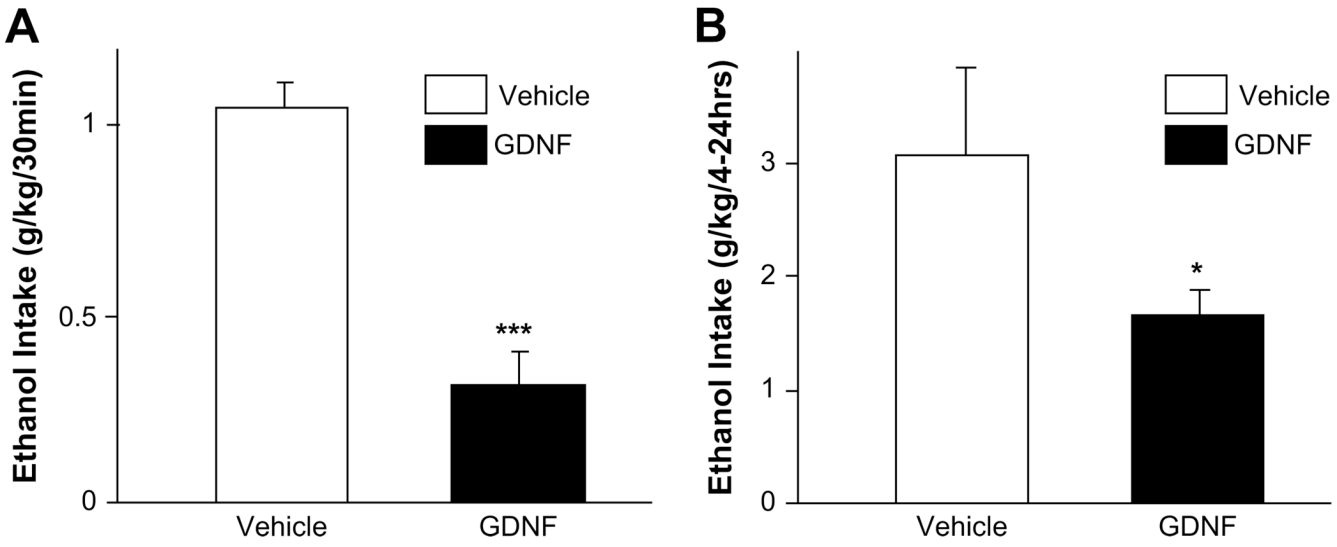

Fig. 4.

Intra-ventral tegmental area microinjection of glial cell line–derived neurotrophic factor (GDNF) induces a rapid and sustained reduction of ethanol consumption. GDNF (10 μg/side) or vehicle was microinjected into the ventral tegmental area 10 min before a 24-h ethanol-drinking session. Mean ± standard error of the mean of ethanol consumed during the first 30 min (A) and the last 20 h (B) of the drinking session, $n = 7$; *$P < .05$; ***$P < .001$ compared to vehicle.