

Autoregulation of glial cell line-derived neurotrophic factor expression: implications for the long-lasting actions of the anti-addiction drug, Ibogaine

Dao-Yao He,* and Dorit Ron*,†,1

*Ernest Gallo Research Center, †Department of Neurology, University of California, San Francisco, Emeryville, California, USA

ABSTRACT We recently showed that the up-regulation of the glial cell line-derived neurotrophic factor (GDNF) pathway in the midbrain, is the molecular mechanism by which the putative anti-addiction drug Ibogaine mediates its desirable action of reducing alcohol consumption (1). Human reports and studies in rodents have shown that a single administration of Ibogaine results in a long-lasting reduction of drug craving (humans) and drug and alcohol intake (rodents). Here we determine whether, and how, Ibogaine exerts its long-lasting actions on GDNF expression and signaling. Using the dopaminergic-like SHSY5Y cell line as a culture model, we observed that short-term Ibogaine exposure results in a sustained increase in GDNF expression that is mediated via the induction of a long-lasting autoregulatory cycle by which GDNF positively regulates its own expression. We show that the initial exposure of cells to Ibogaine or GDNF results in an increase in GDNF mRNA, leading to protein expression and to the corresponding activation of the GDNF signaling pathway. This, in turn, leads to a further increase in the mRNA level of the growth factor. The identification of a GDNF-mediated, autoregulatory long-lasting feedback loop could have important implications for GDNF's potential value as a treatment for addiction and neurodegenerative diseases.—He, D.-Y., Ron, D. Autoregulation of glial cell line-derived neurotrophic factor expression: implications for the long-lasting actions of the anti-addiction drug, Ibogaine. *FASEB J.* 20, E1820–E1827 (2006)

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IBOGAINE IS A PSYCHOACTIVE indole alkaloid extracted from the root bark of the African shrub *Tabernanthe iboga* that has been used for decades in Africa in folklore medicine and rituals. Human anecdotal reports and several studies indicated that Ibogaine reduces craving and withdrawal symptoms for multiple drugs of abuse, including heroine, psychostimulants and cocaine (2–4). The potential anti-addictive properties of Ibogaine were confirmed in rodent models: Ibogaine was shown to attenuate cocaine, nicotine, morphine, heroine, and alcohol-seeking behaviors (5).

For example, Ibogaine was found to reduce cocaine and morphine self-administration (6–8). The drug was also found to alleviate morphine withdrawal symptoms (9). Finally, we and others reported that systemic administration of Ibogaine reduces voluntary ethanol intake in a 2-bottle choice paradigm (1, 10, 11). In addition, we recently reported that Ibogaine inhibits operant ethanol self-administration in rats and reduces ethanol intake in an ethanol reinstatement paradigm (1). Despite its potential use as an anti-addiction agent, Ibogaine is not used in the US to treat addiction because of its undesirable side effects, which include hallucination, bradycardia, and tremor (5). In addition, administration of high concentrations of Ibogaine in rats produced cerebellar Purkinje cell death (12, 13).

In an attempt to separate the desirable anti-addiction actions of the drug from the undesirable side effects, we set out to identify the molecular mechanism mediating Ibogaine's effects on voluntary ethanol consumption. We found that systemic administration of Ibogaine increased the expression of GDNF in the dopaminergic ventral tegmental area (VTA), as well as in a dopaminergic-like cell culture model, the SHSY5Y cell line (1). Furthermore, we found that in SHSY5Y cells, Ibogaine incubation resulted in a time- and dose-dependent activation of the GDNF signaling pathway. When the GDNF pathway was inhibited in the VTA, Ibogaine was significantly less effective in reducing ethanol intake (1). Finally, similar to Ibogaine, GDNF administered into the VTA reduced ethanol consumption (1). Together, our results suggest that up-regulation of the GDNF pathway in the VTA mediates the Ibogaine-induced reduction in voluntary ethanol consumption.

Anecdotal reports have suggested that a single treatment of Ibogaine reduced drug craving in humans for a period of weeks or even up to six months (14). These possible long-lasting actions of Ibogaine have also been reported in animal studies. Single or multiple injections of the drug produced a long-lasting reduction of cocaine self-administration (7, 8), and we observed that a single systemic injection of Ibogaine in rats reduced

¹ Correspondence: 5858 Horton St., Ste. 200, Emeryville, CA, 94608, USA. E-mail: dorit.ron@ucsf.edu
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ethanol consumption for up to 48 h after injection (1). We also found that incubation of SHSY5Y cells with Ibogaine produced sustained changes in the *GDNF* pathway; Ibogaine-mediated increase in *GDNF* expression and secretion, as well as the subsequent phosphorylation (and thus, activation) of the *GDNF* receptor, Ret, lasted for at least 12 h (1). We hypothesized that the long-lasting actions of Ibogaine are mediated, at least in part, via initiation of prolonged activation of the *GDNF* pathway. To test this hypothesis we used SHSY5Y cells as a model system and found that the initial activation of the *GDNF* pathway by Ibogaine leads to the induction of a cycle in which secreted *GDNF* induces expression of itself, leading to the prolonged action of Ibogaine on this signaling pathway.

MATERIALS AND METHODS

Materials

Ibogaine-HCl, phosphatidylinositol phospholipase C (PI-PLC) and cycloheximide (CHX) were purchased from Sigma. Human *GDNF*, anti-phospho-tyrosine antibodies (anti-pTyr), and pUSE vector were purchased from Upstate Cell Signaling Solutions. Anti-*GDNF* monoclonal neutralizing antibodies were purchased from R&D Systems. The inhibitors U0126 and actinomycin D (A/D) were purchased from Calbiochem. The protease inhibitor cocktail was purchased from Roche Applied Science. Anti-Ret antibodies were purchased from Santa Cruz Biotechnology. pGEM-T vector, Reverse Transcription System and 2× polymerase chain reaction (PCR) Master Mix were purchased from Promega Corp. Lipofectamine 2000, Geneticin (G418) and protein G agarose were purchased from Invitrogen. Primers for PCR were synthesized by Sigma-Genosys.

Cell culture

SHSY5Y human neuroblastoma cells were cultured according to the method described in He *et al.* (1). All experiments were carried out in cells that were incubated in a low serum medium containing 1% FBS for 2 days.

pUSE-*GDNF* stable cell line

Human *GDNF* cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of SHSY5Y cells, and cloned into pGEM-T vector. *GDNF* primers were as follows: upstream 5'-G AAG CTT ATG AAG TTA TGG GAT GTC GTG GCT GTC-3' and downstream 5'-AAG CTT CTC GAG TCA GAT ACA TCC ACA CCT TTT AGC-3'. pGEM-*GDNF* was digested with restriction endonucleases *Hind*III and *Xho*I to produce the *GDNF* cDNA insert, which was then recombined into pUSE vector. The integrity of the *GDNF* cDNA was confirmed by sequencing. Lipofectamine 2000 was used to transfect pUSE-*GDNF* (pGDNF) and pUSE empty vector (control) into SHSY5Y cells, the transfected cells were maintained in growth media containing 800 µg/ml G418 and were subcultured every 5–6 days. Stable cells expressing pGDNF or pUSE were obtained after a one-month culture selection, then maintained in growth media containing 500 µg/ml G418. Prior to experiments, the pGDNF cells and the pUSE cells were grown in media without G418 for 2 days and incubated in low serum media containing 1% FBS

for another 2 days. Conditioned media was collected from an overnight culture (15 h) of pGDNF cells (CM-GDNF). The pGDNF cells overexpressed *GDNF* (Supplemental Fig. S1A) and secreted high levels of the *GDNF* polypeptide (Supplemental Fig. S1B). These cells maintained constitutively high levels of Ret (Supplemental Fig. S2A) and ERK (Supplemental Fig. S2B) phosphorylation.

Treatments

Ibogaine was dissolved in water to make a stock solution of 10 mM and was used at a final concentration of 10 µM as described in He *et al.* (1). Cells were incubated with Ibogaine for the indicated time periods. To study the persistence of Ibogaine's effects following washout, cells were treated with Ibogaine for 3 h, washed, and incubated in fresh media for the indicated times, as shown in the figure legends. PI-PLC was used to hydrolyze GFRα1 from the cell surface; treatment involved incubation of cells with 0.3 u/ml PI-PLC for 1 h followed by washes with fresh media before treatment with conditioned media. Anti-*GDNF* neutralizing antibodies (anti-*GDNF* antibodies) were dissolved in PBS as a stock solution of 500 µg/ml, and were used at a concentration of 10 µg/ml.

Reverse transcription-polymerase chain reaction

Cells were treated in 6-well plates followed by total RNA isolation using 1 ml/well of Trizol reagent, as described in the manufacturer's protocol. The RNA samples were dissolved in 40 µl of water. RT-PCR was then performed to analyze *GDNF* expression level, with *Actin* as an internal control. Briefly, 1–2 µg of total RNA was used in a 10 µl reverse transcription (RT) reaction, using the oligo(dT)15 primer and the Reverse Transcription System kit, except in experiments with the inhibitors A/D and CHX. In those experiments, 4 µl of each RNA sample was used in the 10 µl RT reaction. The RT reaction was carried out at 42°C for 30 min followed by heating at 99°C for 6 min, then diluted to a final volume of 50 µl. 6 µl of the RT product was used for PCR in a 40 µl PCR mix; the PCR reaction was run for 35 cycles using *GDNF* primers or for 30 cycles using *Actin* primers. In parallel, PCR was performed with *GDNF* and *Actin* primers using the RNA samples as the template to confirm that results were not due to genomic DNA contamination. Signals of PCR products were visualized by electrophoresis in Tris/acetic acid/EDTA (TAE) buffer containing 0.25 mg/ml ethidium bromide, photographed by Eagle Eye II (Stratagene, La Jolla, CA, USA), and quantified by NIH Image 1.61.

Immunoprecipitation

Cells were treated in T75 flasks and lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, protease inhibitor complete minitab and 10 mM sodium orthovanadate). 500 µg of homogenate was incubated with 5 µg anti-Ret antibodies in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) overnight at 4°C, followed by 2 h incubation with Protein G agarose. Samples were separated on an SDS-PAGE gel for Western blot analysis with anti-pTyr and anti-Ret antibodies.

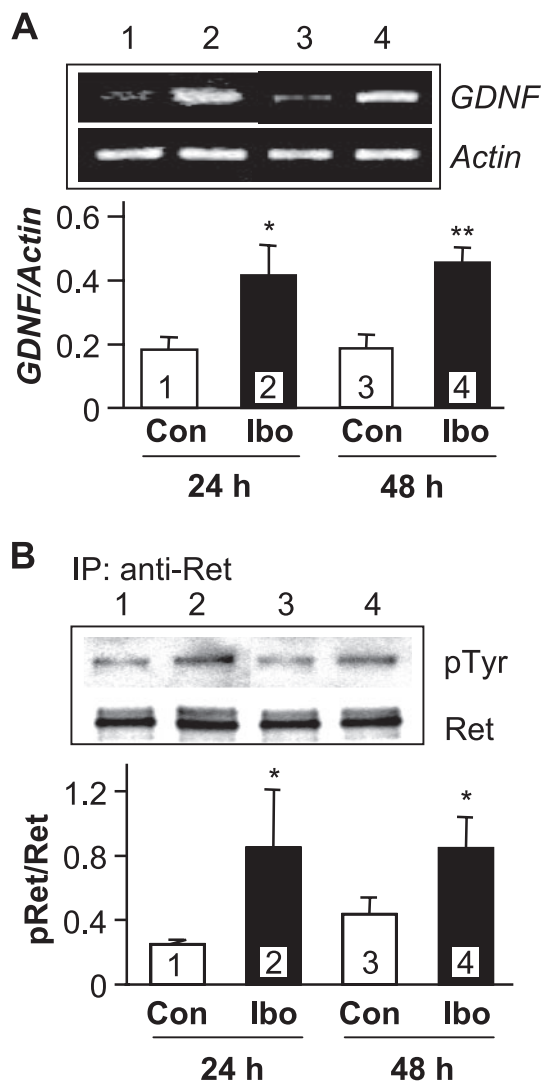


Figure 1. Ibogaine induces long-lasting expression and signaling activity of GDNF. SHSY5Y neuroblastoma cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing 1% FBS for 2 days, then treated without (Con, lanes 1 and 3) or with (Ibo, lanes 2 and 4) 10 μ M Ibogaine for 24 h and 48 h. **A)** *GDNF* expression was analyzed by RT-PCR with *Actin* as control. Histogram depicts the mean ratios of *GDNF* to *Actin* $\pm$ sd of three experiments. **B)** Ret was immunoprecipitated using anti-Ret antibodies, followed by immunoblotting with anti-p-Tyr and anti-Ret antibodies. Histogram depicts the mean ratios of pTyr (pRet) to Ret $\pm$ sd of five experiments. * P < 0.05; ** P < 0.01, compared with control.

RESULTS

Ibogaine treatment induces a persistent increase in GDNF expression and Ret phosphorylation

First, we tested the effect of long-term incubation of SHSY5Y cells with Ibogaine on the expression of *GDNF*, and on the activation of the GDNF receptor, Ret. Treatment of cells with Ibogaine resulted in a sustained increase in *GDNF* mRNA for 24 and 48 h (Fig. 1A, lanes 2, 4), as well as an increase in Ret phosphorylation (and

thus, activation), which also persisted for 48 h after Ibogaine incubation (Fig. 1B, lanes 2, 4).

Next, we determined whether an acute treatment with Ibogaine leads to a sustained increase in *GDNF* mRNA levels. Cells were treated with Ibogaine for 3 h, at which time media containing the drug was removed, the cells were washed extensively, and new, Ibogaine-free media was added for the indicated times (Fig. 2A). We found that 3 h incubation of cells with Ibogaine resulted in a persistent increase in *GDNF* mRNA levels even 12 h after Ibogaine removal (Fig. 2A). Next, we tested whether the long-term increase in *GDNF* message after acute exposure of cells to the drug was due to an increase in the stability of *GDNF* mRNA. Cells were treated with Ibogaine for 3 h, washed, and fresh media was added with or without actinomycin D (A/D), an inhibitor of transcription. As shown in Fig. 2B, A/D inhibited the long-lasting increase in *GDNF* mRNA, suggesting that Ibogaine does not affect the stability of the growth factor's message. We also tested whether the initial increase in *GDNF* expression on Ibogaine exposure, which leads to activation of the GDNF pathway (1), is required for the long-lasting increase in *GDNF* mRNA levels. To test this possibility, cells were treated with Ibogaine for 3 h and subsequently, following Ibogaine washout, the cells were incubated with fresh media containing anti-GDNF neutralizing antibodies. Incubation of cells with anti-GDNF antibodies, which sequester GDNF and thus inhibit the GDNF signaling pathway, abolished the increase in the expression of *GDNF* (Fig. 2C). These results suggest that Ibogaine triggers the long-lasting increase in *GDNF* expression in a GDNF-dependent manner.

Autoregulation of GDNF expression

Neurotrophins such as the brain-derived neurotrophic factor (BDNF) have been reported previously to self-regulate their expression and secretion (15–17). We, therefore, speculated that Ibogaine mediates its long-lasting activities via autoregulation of GDNF expression and function. First, we tested the effects of GDNF on its own transcription. We found that incubation of SHSY5Y cells with recombinant GDNF induced a sustained increase in *GDNF* expression (Fig. 3A), suggesting a positive feedback mechanism in which activation of the GDNF pathway results in an increase in the message of the growth factor itself. To confirm this possibility, we incubated cells stably expressing the empty pUSE vector with conditioned media from cells that stably overexpress GDNF and secrete high levels of the growth factor (CM-GDNF; see Supplemental Fig. 1), to test whether a prolonged increase in *GDNF* mRNA was observed. As shown in Fig. 3B, incubation of cells with CM-GDNF induced a continuous increase in *GDNF* expression. This increase was not observed when the same cells were incubated with media of cells expressing the empty vector pUSE (Fig. 3B, CM-C). Next, we determined whether the induction of *GDNF* expression requires the ligation of the secreted

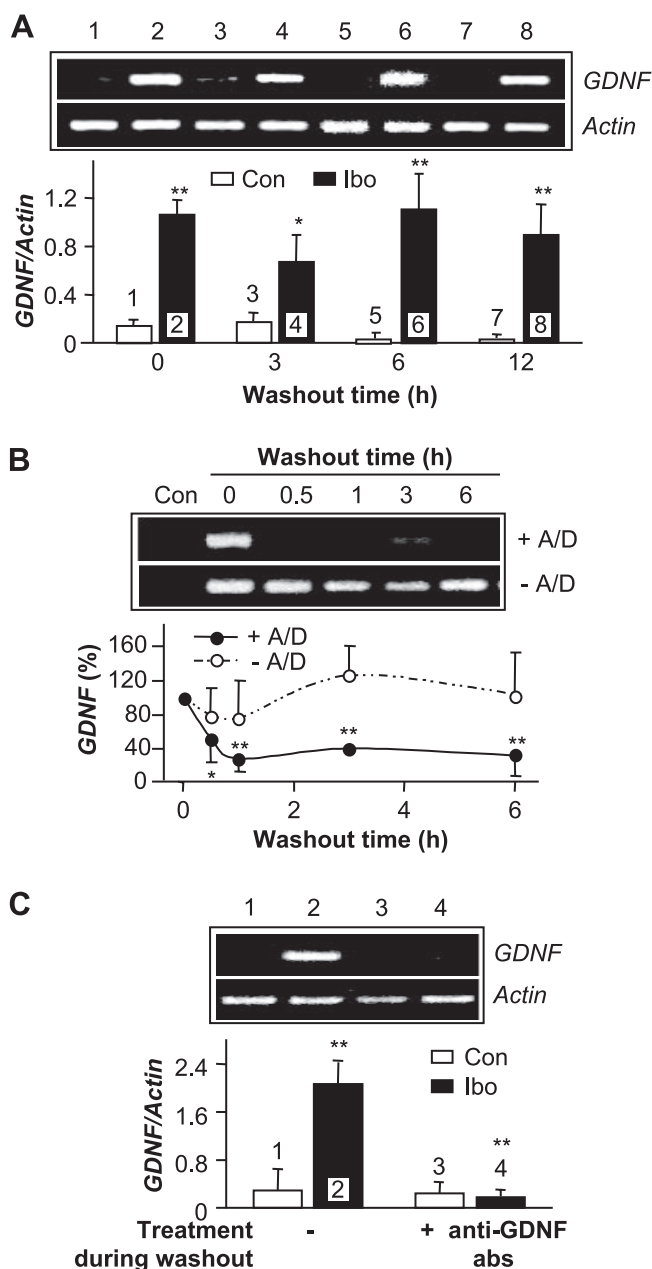


Figure 2. 3 h treatment with Ibogaine leads to a long-lasting increase in *GDNF* expression that is mediated by the *GDNF* polypeptide. *A*) Cells were treated without (Con, lanes 1, 3, 5, and 7), or with (lanes 2, 4, 6, and 8) 10 μ M Ibogaine for 3 h. Cells were then extensively washed and incubated in fresh media for the indicated time period. *GDNF* expression was analyzed by RT-PCR. Histogram depicts the mean ratios of *GDNF* to *Actin* $\pm$ sd of three experiments. * $P < 0.05$; ** $P < 0.01$, compared with control. *B*) Cells were treated without (Con) or with 10 μ M Ibogaine for 3 h, then washed extensively and incubated in fresh media for the period indicated without (lower panel) or with (upper panel) 5 μ g/ml actinomycin D (A/D). Histogram depicts the mean percentages of *GDNF* $\pm$ sd of three experiments. * $P < 0.05$; ** $P < 0.01$, compared with 0 time. *C*) Cells were treated without (lanes 1 and 3), or with (lanes 2 and 4) 10 μ M Ibogaine for 3 h. Cells were then extensively washed and incubated with fresh media in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 μ g/ml anti-*GDNF* neutralizing antibodies for 3 h. Histogram depicts the mean ratios of *GDNF* to *Actin* $\pm$ sd of three experiments. ** $P < 0.01$, lane 2 vs. 1, or lane 4 vs. 2.

polypeptide to its receptors. Cells were, therefore, treated with PI-PLC, which hydrolyzes the glycosylphosphatidylinositol (GPI) link of the *GDNF* coreceptor GFR α 1 and thus blocks *GDNF* signaling, or anti-*GDNF* neutralizing antibodies, followed by addition of CM-*GDNF*. As shown in Fig. 3C, D (lanes 2 vs. 4), both the inhibitory reagents prevented the increase in *GDNF* expression. In addition, similar to Ibogaine's effect, short treatment with the CM-*GDNF* induced a persistent increase in the message of the growth factor, even after the CM-*GDNF* was removed (Fig. 3E). Finally, we found that, similar to the long-lasting actions of Ibogaine, the long-term increase in the expression of *GDNF* induced by CM-*GDNF* was inhibited in the presence of A/D, suggesting that *GDNF* does not increase the stability of its own message (Fig. 3F).

Ibogaine- and *GDNF*-induced *GDNF* expression are mediated via the activation of MAP kinase

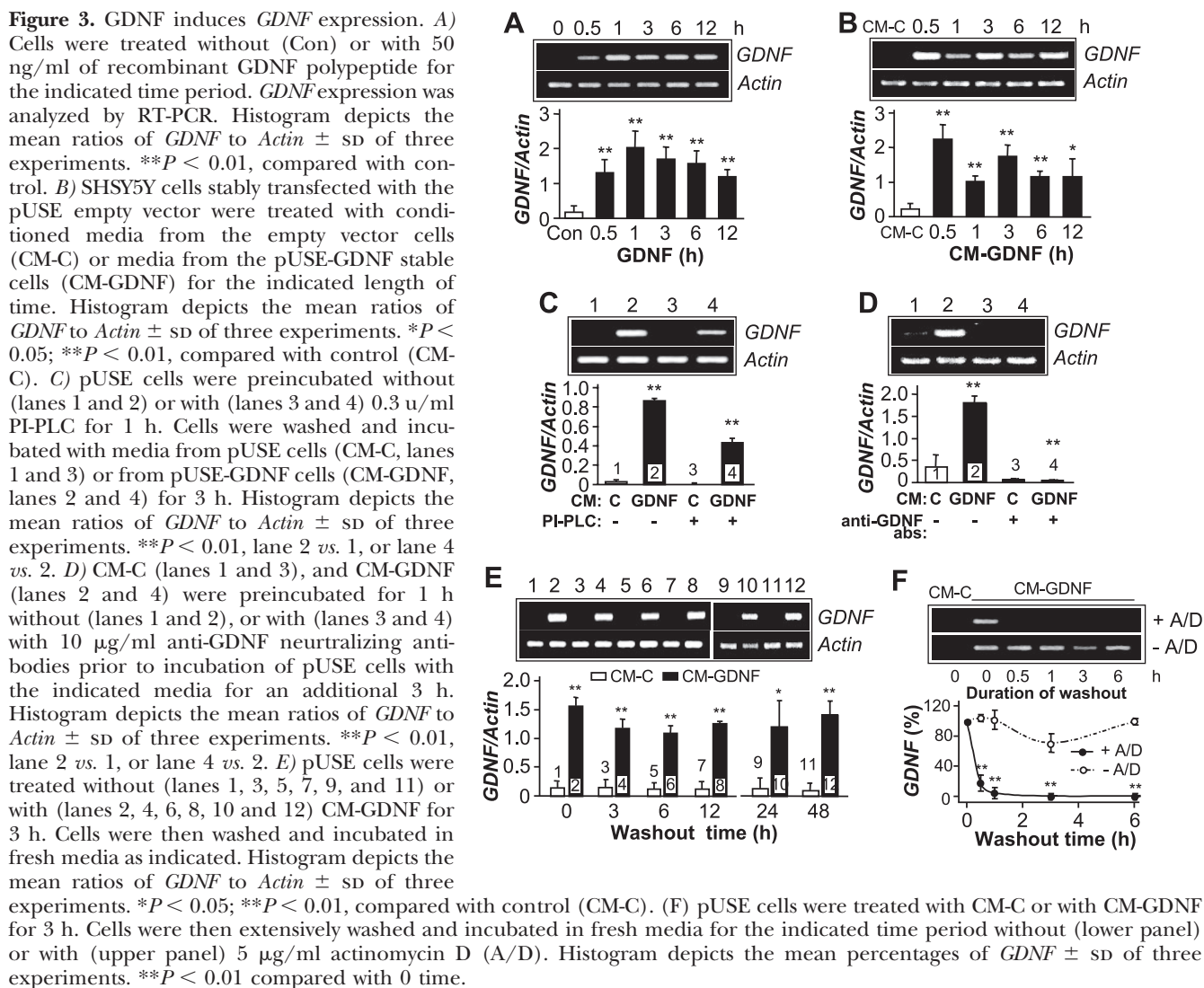
GDNF-mediated autophosphorylation of the Ret receptor initiates activity in several different downstream signaling pathways, including activation of the mitogen-activated protein (MAP) kinases (ERKs), leading to alteration in gene expression (18). To determine whether activation of the MAP kinase pathway is required for Ibogaine-mediated long-term induction of *GDNF* mRNA, we investigated the effects of Ibogaine and CM-*GDNF* on *GDNF* expression in cells that were preincubated with the MEK-specific inhibitor U0126. We found that blockade of ERK activity by U0126 inhibited the induction of *GDNF* expression in cells given 3 h exposure to either Ibogaine (Fig. 4A) or CM-*GDNF* (Fig. 4B); this effect was also observed when the MEK inhibitor was added after Ibogaine washout (Fig. 4C).

Long-term but not short-term up-regulation of *GDNF* expression depends on protein synthesis

Our data suggest that acute exposure of cells to Ibogaine results in an increase in *GDNF* mRNA levels, followed by subsequent translation of the polypeptide, which is then secreted to up-regulate its own message, initiating a *GDNF*-mediated autoregulatory cycle. If our model is correct, then increases in *GDNF* message on short-term Ibogaine exposure should not be dependent on protein synthesis. As predicted, a brief (0.5 h) incubation of cells with Ibogaine was insensitive to the protein synthesis inhibitor, cycloheximide (Fig. 5A, lane 3; Fig. 5B, lane 3), whereas longer (≥ 1 h) Ibogaine-mediated induction of *GDNF* expression was sensitive to cycloheximide treatment (Fig. 5A, lanes 5, 7, 9; Fig. 5B, lane 6).

DISCUSSION

Based on our results, we propose a model (Fig. 6) in which Ibogaine exposure leads to an increase in *GDNF*



message, followed by the translation and subsequent secretion of the polypeptide, resulting in the activation of the GDNF receptor tyrosine kinase Ret, which then activates the MAP kinase pathway to further up-regulate the message of the growth factor. This GDNF-mediated autoregulatory positive feedback mechanism may explain the long-lasting actions of Ibogaine to reduce drug and alcohol self-administration, which have been shown in rodent models to last 24 h or longer (1, 6, 7). In addition, this mechanism may account for anecdotal human reports suggesting that a single treatment of Ibogaine reduces craving for various drugs of abuse for up to six months (14); however, these observations need further investigation.

Autoregulation of growth factors has been previously documented. For example, BDNF has been reported to positively regulate its own expression (17), secretion (15), and dendritic targeting of its own mRNA (16). In addition, various endogenous and pharmacological agents have been shown to control *GDNF* expression (19–21); however, to our knowledge this is the first report of the up-regulation of *GDNF* expression via GDNF itself. The mechanism for GDNF regulation of

its own message needs to be determined, however, analysis of the promoter region of the human *GDNF* gene revealed putative Sp-1 and activating protein (AP)-2 transcription factor binding sites (22). The MAP kinase pathway has been shown to up-regulate the transcription of another growth factor, the vascular endothelial growth factor (VEGF), via the Sp1 and AP-2 binding sites (23). We found that the autoregulatory increase in *GDNF* expression is inhibited on incubation of cells with a MEK inhibitor, suggesting that activation of the MAP kinase pathway contributes to the long-lasting increase in *GDNF* expression. Therefore, it is possible that the Sp1 and AP-2 transcription factor binding sites within the *GDNF* promoter contribute to a GDNF-mediated increase in its own mRNA.

An intriguing possibility is that autoregulation of GDNF expression and sustained activation of the GDNF pathway contribute to such long-term processes as neuronal survival, as GDNF has been shown to be a critical mediator of the development and survival of midbrain dopaminergic neurons (18). This positive cycle may also account for GDNF's actions on dopamine synthesis (24) via increasing the phosphorylation

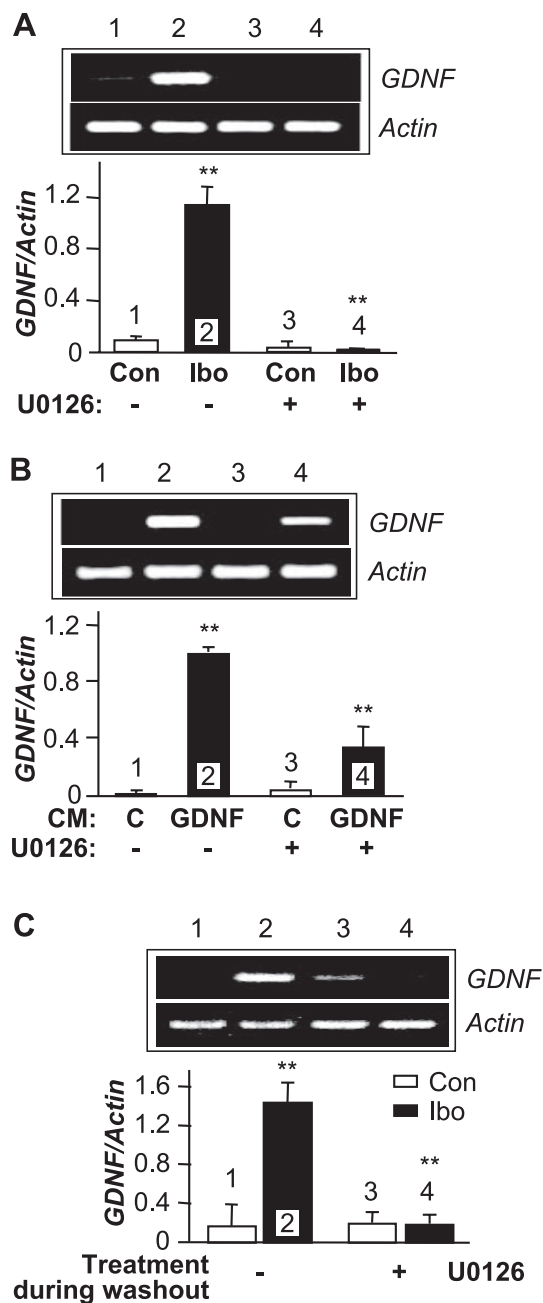


Figure 4. Ibogaine- and GDNF-induced *GDNF* expression are mediated via the MAP kinase pathway. *A*, *B*) Wild-type (WT) SHSY5Y cells (*A*) or pUSE cells (*B*) were preincubated without (lanes 1, 2) or with (lanes 3, 4) 20 μ M U0126 for 0.5 h before the addition of 10 μ M Ibogaine (*A*, lanes 2, 4) or CM-GDNF (*B*, lanes 2, 4) for 3 h. *C*) WT SHSY5Y cells were treated without (lanes 1, 3) or with (lanes 2, 4) 10 μ M Ibogaine for 3 h. Cells were then washed and incubated in fresh media in the absence (lanes 1, 2) or presence (lanes 3, 4) of 20 μ M U0126 for 3 h. Histogram depicts the mean ratios of *GDNF* to *Actin* $\pm$ SD of three experiments. ** P < 0.01, lane 2 vs. 1, or lane 4 vs. 2.

and activity of tyrosine hydroxylase, the enzyme controlling the rate-limiting step in dopamine biosynthesis (25). Finally, GDNF has been shown to contribute to synaptic plasticity processes and learning and memory (26–29). One of the intracellular signaling cascades

initiated via GDNF-mediated activation of the Ret receptor is the MAP kinase pathway (18), and MAP kinase has been shown to be a critical player in long-term potentiation and learning and memory (30–32). Together our results suggest a mechanism for GDNF involvement in synaptic plasticity.

Importantly, this autoregulatory positive feedback in the GDNF pathway may have implications for the treatment of addiction. Various studies have suggested that GDNF acts as a negative regulator of biochemical and behavioral adaptations to drugs of abuse and alcohol. For example, infusion of GDNF into the VTA of rats blocks and/or reverses cocaine-induced increases in the NR1 subunit of the NMDA receptor, alters Δ FosB and PKA Ca in the nucleus accumbens, and blocks the behavioral effects of repeated exposure to cocaine, as measured by the conditioned place preference procedure (33). Green-Sadan *et al.* reported that transplantation of simian virus-40 glial cells, which produce and secrete GDNF, or delivery of GDNF-conjugated nanoparticles into the dorsal and ventral striatum impaired the acquisition of cocaine self-ad-

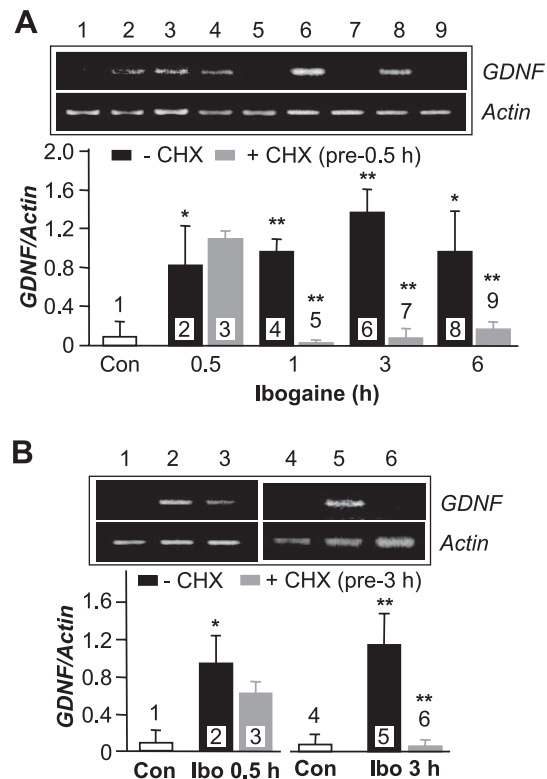


Figure 5. Long-lasting Ibogaine-mediated induction of *GDNF* expression depends on protein synthesis. *A*) SHSY5Y cells were preincubated without (lanes 1, 2, 4, 6, and 8) or with (lanes 3, 5, 7, and 9) 30 μ g/ml cycloheximide (CHX) for 0.5 h before the addition of 10 μ M Ibogaine (lanes 2–9) for the indicated time periods. *B*) Cells were preincubated without (lanes 1, 2, 4, and 5) or with (lanes 3 and 6) 30 μ g/ml CHX for 3 h before the addition of 10 μ M Ibogaine for 0.5 h (lanes 2 and 3) or for 3 h (lanes 5 and 6). Histogram depicts the mean ratios of *GDNF* to *Actin* $\pm$ SD of 3 experiments. * P < 0.05; ** P < 0.01, Ibogaine alone (–CHX) vs. control, or + CHX vs. –CHX.

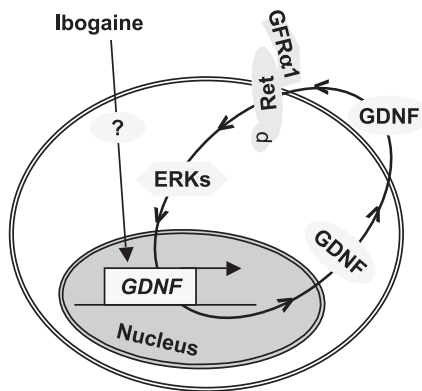


Figure 6. Diagram of Ibogaine- and GDNF-mediated long-lasting induction of GDNF expression and signaling. The data presented suggest a model in which GDNF or Ibogaine up-regulates *GDNF* expression leading to the translation of the polypeptide, which is secreted and consequently activates the Ret receptor and its downstream target ERK. This, in turn, results in further increases in *GDNF* expression. This positive feedback loop induces a sustained long-lasting activation of the pathway.

ministration (34, 35). Finally, we found that intra-VTA infusion of GDNF reduced rats' operant self-administration of ethanol (1). Taken together, these results suggest that agents which activate the GDNF pathway and/or increase GDNF message may be useful drugs to treat addiction, and our current work implies that short-term treatment with such agents may result in long-lasting changes in addictive phenotypes. Finally, the identification of a GDNF-mediated autoregulatory feedback loop may have implications for its potential therapeutic value as treatment for neurodegenerative diseases such as Parkinson's disease. **[F]**

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