GDNF acutely modulates excitability and A-type K^+ channels in midbrain dopaminergic neurons

Feng Yang[1,2], Linyin Feng[1,2], Fang Zheng[3], Steven W. Johnson[3], Jing Du[2], Liya Shen[4], Chien-ping Wu[1] and Bai Lu[2]

[1] *Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China 200031*

[2] *Unit on Synapse Development and Plasticity, LCSN, NICHD, NIH, Bethesda, Maryland 20892-4480*

[3] *Department of Physiology and Pharmacology, Oregon Health Science University, Portland, Oregon 97201*

[4] *Laboratory of Carcinogenesis and Tumor Promotion, NCI, NIH, Bethesda, Maryland 20892-4255*

Correspondence should be addressed to B.L. (lub@codon.nih.gov)

Published online: 9 October 2001, DOI: 10.1038/nn734

Glial cell line-derived neurotrophic factor (GDNF) prevents lesion-induced death of midbrain dopaminergic neurons, but its function in normal brain remains uncertain. Here we show that GDNF acutely and reversibly potentiated the excitability of cultured midbrain neurons by inhibiting transient A-type K^+ channels. The effects of GDNF were limited to large, tyrosine hydroxylase (TH)-positive dopaminergic neurons, and were mediated by mitogen associated protein (MAP) kinase. Application of GDNF also elicited a MAP kinase-dependent enhancement of the excitability in dopaminergic neurons in midbrain slice. These results demonstrate an acute regulation of GDNF on ion channels and its underlying signaling mechanism, and reveal an unexpected role of GDNF in normal midbrain dopaminergic neurons.

GDNF belongs to a family of neurotrophic factors that regulate not only the development and function of the nervous system but also that of the kidney and the gastrointestinal system[1,2]. Binding of GDNF to the GPI-linked receptor GFR-α1 leads to activation of c-RET tyrosine kinase[3–6]. Subsequently, a number of downstream signaling pathways are activated, including MAP kinase and PI3 kinase[7,8].

The most extensively studied effect of GDNF is the regulation of survival of dopaminergic neurons in the midbrain substantia nigra. GDNF was originally identified as a potent trophic factor for cultured midbrain dopaminergic neurons[9]. Subsequent studies demonstrate that GDNF is capable of attenuating the death of the nigra neurons in a variety of lesion models *in vivo*. For example, GDNF prevents and reverses the degeneration of nigra dopaminergic neurons induced by many chemical insults, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[10,11], 6-hydroxy-dopamine[12,13] and methamphetamine[14]. It also protects nigra neurons from death induced by mechanical lesion of medial forebrain bundle[15]. Therefore, GDNF is considered as a potential therapeutic agent for Parkinson's disease[16,17]. Although substantial evidence suggests that GDNF enhances the survival of injured neurons, its function on normal dopaminergic neurons in the intact animals remains unclear.

Studies of the neurotrophin family of proteins have revealed two modes of action: acute modulation of ion channels and synaptic transmission, and long-term regulation of cell survival and differentiation[18,19]. GDNF exerts a wide range of long-term effects on diverse populations of peripheral and central neurons ranging from cerebellum Purkinje cells to sympathetic neurons[1,2,20,21]. Spinal cord motoneurons seem to be another major target of GDNF regulation; the number of motoneurons is selectively reduced in mice lacking GDNF[22]. GDNF also promotes the long-term development of the neuromuscular syna pses[23,24]. However, unlike neurotrophins, little is known about the acute effect of GDNF.

Here we investigated the acute effects of GDNF on the function of midbrain dopaminergic neurons. Surprisingly, we found a rapid and reversible modulation of A-type potassium channels by GDNF, leading to a potentiation of the excitability of these neurons. Furthermore, we showed that the acute effects of GDNF are mediated through a mechanism that involves activation of MAP kinase. Thus, we revealed an acute modulation of ion channels by GDNF. Moreover, our results suggest that the major function of GDNF in normal, undamaged midbrain dopaminergic neurons may be to regulate neuronal excitability, and consequently, dopamine release.

Results

To determine the acute effect of GDNF, we perfused GDNF (25 ng/ml) directly to isolated midbrain neurons in culture and measured the changes in their physiological properties using whole-cell, current- or voltage-clamped recordings. To reduce the complexity of GDNF effects, we included Cd^{2+} (0.4 mM) in the perfusion Ringer's solution to block all Ca^{2+} channels and Ca^{2+}-activated K^+ channels. Application of GDNF elicited a small but reproducible depolarization (**Fig. 1a**, **Table 1**), possibly

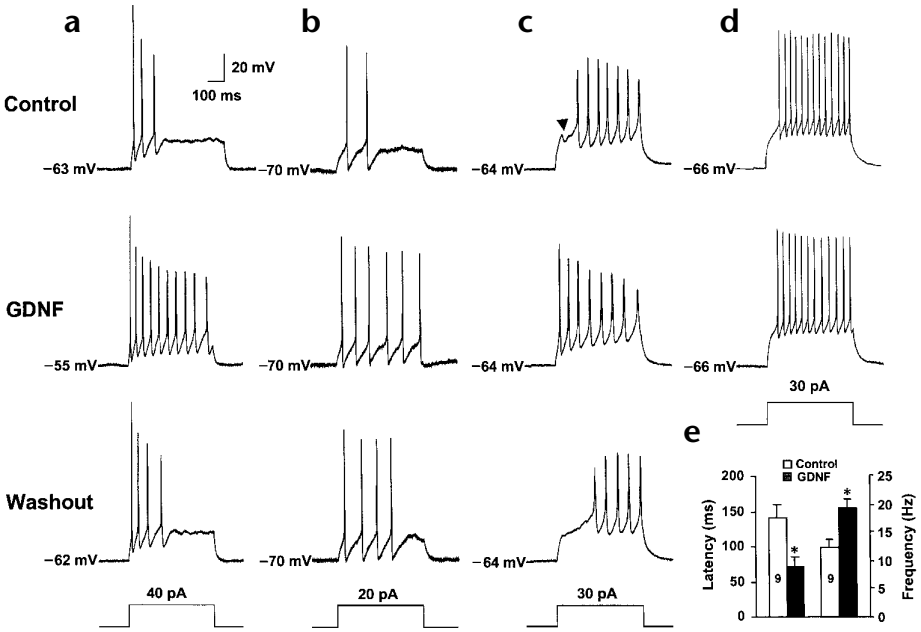

Fig. 1. GDNF modulation of membrane potential and firing behavior of cultured midbrain neurons. A supra threshold depolarizing current was injected into a midbrain neuron, and changes in membrane potentials were recorded under the current-clamp mode. GDNF (25 ng/ml) was perfused in and washed out of the cultures through a bath perfusion system. (**a**) Acute application of GDNF elicited membrane depolarization. (**b**, **c**) Membrane potential during GDNF application was corrected to the original resting membrane potential with current injection to better reveal the effects of GDNF. (**b**) GDNF increased the spike frequency, and reduced the first spike latency. (**c**) Application of GDNF eliminated the notch-like repolarization during depolarization in some neurons. (**d**) GDNF had no effect on small neurons that fire at high frequency. (**e**) GDNF increased the spike frequency and reduced the first spike latency in the absence of Cd^{2+}. Number of recordings are indicated in the columns. *Significantly different from control (paired *t*-test, $p < 0.05$).

by modulating leakage current (I_L) or hyperpolarization-activated cation current (I_H). To examine other effects of GDNF besides depolarization, the resting membrane potentials during GDNF application were corrected to the originals by current injection in all remaining experiments. A depolarizing current step injected into a current-clamped neuron usually induces a train of action potentials, or repetitive spikes (**Fig. 1**). Application of GDNF, but not vehicle (Ringer's solution containing the same blockers), consistently elicited an increase in spike frequency, defined as the inverse of the interval between the first two spikes (**Fig. 1b**). Quantitative analysis of all neurons recorded indicated that spike frequency increased by 36.5% (**Table 1**), revealing an excitatory effect of GDNF in the absence of depolarization. GDNF also reduced the first spike latency, the time needed from current injection to first spike appearance (**Fig. 1b**, **Table 1**). Some neurons exhibited a notch-like repolarization before the first spike (**Fig. 1c**). Application of GDNF eliminated such repolarization. On average, the increase in firing began about 3 minutes after application of GDNF, and reached the plateau in about 10 minutes (data not shown).

Table 1. Effect of GDNF on membrane properties of cultured midbrain neurons.

	Control	GDNF	*n*
Resting potential (mV)	68.0 ± 6.5	60.9 ± 7.2*	61
Input resistance (GΩ)	1.34 ± 0.44	1.57 ± 0.48*	52
Action potential threshold (mV)	−35.3 ± 10.2	−32.5 ± 13.2	61
Action potential amplitude (mV)	89.8 ± 12.8	92.6 ± 12.2	61
First spike latency (ms)	132.9 ± 86.1	71.2 ± 65.4*	61
Spike frequency (Hz)	12.6 ± 3.7	17.2 ± 5.1*	61
Maximal conductance (nS)	12.7 ± 1.1	6.3 ± 0.8	14

Action potential threshold, the first point on the rising phase of the spike where the rate of rise exceeded 50 mV/ms (ref. 50). First spike latency, the time needed from current injection to first spike appearance. Spike frequency, the inverse of the interval between the first two spikes. Maximal conductance, a channel conductance calculated at +80 mV depolarization. g_{max} = peak $I_A/(V_{test} - V_{rev})$, where V_{test} = 80 mV and V_{rev} = −96 mV. Values given are mean ± s.d. Paired Student's *t*-test was used. *$p < 0.01$.

Control experiments were done to determine the specificity of the GDNF effects. Application of heat-inactivated GDNF (25 ng/ml) did not elicit any of these effects (data not shown). GDNF did not affect the action potential threshold or amplitude (**Table 1**). Perfusion of the Ringer's solution after GDNF application restored spike frequency, increased the first spike latency and recovered notch-like repolarization, suggesting that these GDNF effects are reversible (**Fig. 1**). Although most of our current clamp recordings were done in the presence of Cd^{2+}, GDNF still potentiated neuronal firings in the absence of Cd^{2+} (**Fig. 1e**). In these midbrain cultures, there are several different types of neurons[26]. Not all neurons were responsive to GDNF application. Among 208 neurons tested, 61 neurons showed significant responses to GDNF (29.3%). In general, the GDNF-responsive neurons were large, multipolar neurons. These cells fired at lower frequency (<15 Hz) during 20–40 pA step depolarization and showed obvious spike frequency adaptation[27] (**Fig. 1a–c**). In contrast, small midbrain neurons that fire high-frequency spikes (>25 Hz)[27] did not respond to GDNF (**Fig. 1d**).

The identities of the GDNF-responsive neurons were further characterized by double-labeling experiments. Neurons were loaded with fluorescence dye Alexa Fluor 594 through the patch pipette (5 mM) while being tested for their ability to respond to GDNF. Fixatives (2% paraformaldehyde in Ringer's solution) were then perfused into the culture dish for 20 minutes before the patch pipette was gently pulled out. The cultures were washed and processed for immunofluorescence staining using an antibody against tyrosine hydroxylase (TH). Thirteen of fifteen GDNF responsive cells labeled with Alexa Fluor 594 were TH-positive dopaminergic neurons (example, **Fig. 2a**). In contrast, cells that did not respond to GDNF were never stained positive-

ly with the TH antibody. Statistical analysis indicated that the correlation between GDNF-responsive cells and TH-positive cells is highly significant (chi-square test). Thus, the effects of GDNF are restricted to large, TH-positive dopaminergic neurons.

Changes in first-spike latency, spike frequency and notch-like repolarization all suggest alteration in A-type potassium channels[25,28–30]. We therefore performed whole-cell voltage-clamped recording to determine whether GDNF indeed modulates A-current (I_A). Ringer's solution was supplemented with tetrodotoxin (TTX, 1 μM) and $CdCl_2$ (0.4 mM) to block Na^+ and Ca^{2+} channels, respectively. Tetraethylammonium (TEA, 5 mM) was also included to block delayed rectified K^+ channels. A two-step voltage protocol was used to measure I_A (**Fig. 2b**, top). A step depolarization from –30 mV to +50 mV activates most of the residual K^+ channels but not I_A, whereas depolarization from –120 mV to +50 mV activates all potassium currents including I_A. The difference between the two traces represents A-current (**Fig. 2b**). The identity of I_A was further confirmed by application of the A channel blocker 4-aminopyridine (4-AP, 10 mM), which virtually eliminated this current (data not shown). Perfusion of GDNF, but not vehicle, rapidly and markedly reduced I_A (**Fig. 2b**). Of 126 cells studied, 40 showed a significant reduction after GDNF application, and again virtually all of them were large, multipolar neurons.

We next tested whether changes in Na^+ and Ca^{2+} currents could also contribute to the increase in excitability induced by GDNF. For Na^+ current recording, cells were perfused with Ringer's solution containing TEA, 4-AP and Cd^{2+}, but not TTX. A series of step depolarizations (–70 to approximately +20 mV) elicited Na^+ currents of different sizes. Despite the potent effect on I_A, application of GDNF did not elicit any change in Na^+ currents (**Fig. 2c**). Ca^{2+} currents were recorded using a different extracellular solution, containing TEA, 4-AP and TTX, but not Cd^{2+}. Ba^{2+} was used as the charge carrier to avoid Ca^{2+}-induced inactivation. By subtracting currents elicited by two-depolarization protocols (one –80 to +40 mV, the other –60 to +40 mV, both with 10-mV increments), we observed low-voltage activated (LVA) Ca^{2+} currents. High-voltage activated (HVA) Ca^{2+} currents were recorded using the second protocol. Application of GDNF had no effect on LVA Ca^{2+} currents but elicited a small increase in HVA Ca^{2+} currents when membrane potential was depolarized to around 0 mV (**Fig. 2d**). However, this increase could not be involved in GDNF modulation of excitability because most of our studies on excitability were done in the presence of Cd^{2+}, which blocks all Ca^{2+} channels. Thus, the effect of GDNF on neuronal excitability is mediated primarily by the inhibition of A-type K^+ channels.

We then performed detailed pharmacological analyses of the GDNF effect on I_A. Dose–response experiments indicated that I_A was inhibited as much as 40% when the highest concentration of GDNF was used in the perfusion pipette (**Fig. 3a**). The median effective concentration (EC_{50}) was 0.177 ng/ml. To determine whether the GDNF effect was reversible, we perfused the midbrain neurons with GDNF (25 ng/ml) for one minute, followed by a two-minute wait and then perfusion of the Ringer's solution. The effect of GDNF was almost completely abolished after washout with Ringer's solution (**Fig. 3b**). We next constructed current–voltage (*I–V*) curves in which the test voltage was plotted against peak I_A. Application of GDNF clearly shifted the curve to the right (**Fig. 3c**). Heat-inactivated GDNF had no effect. Single exponential fit of the rising phase of I_A revealed that the activation time constants were 3.3 ms when perfused

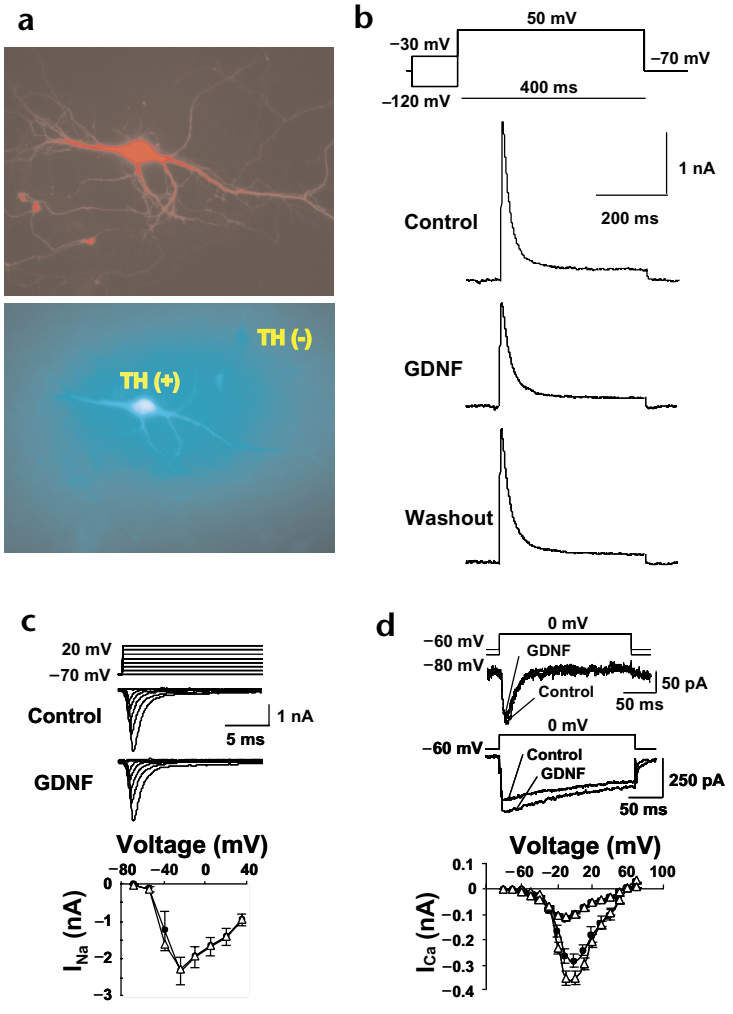

Fig. 2. GDNF suppresses A-type potassium currents (I_A). (**a**) Effect of GDNF is restricted to large, TH-positive dopaminergic neurons. Midbrain neurons were filled with Alexa Fluor 594 (top) using a whole-cell recording electrode while the effect of GDNF was tested. Cells were then fixed and processed for immunocytochemistry using an anti-TH antibody (bottom). (**b**) Examples showing that GDNF reversibly reduces I_A. Na+ currents were blocked by TTX; Ca^{2+} currents and Ca^{2+} activated K^+ currents, by $CdCl_2$; delayed rectified K^+ current (I_k), by TEA. I_A was obtained by subtracting the currents induced by the two voltage protocols shown at top. (**c**) Effect of GDNF on Na^+ currents. Cells were perfused with Ringer's solution containing TEA, 4-AP and $CdCl_2$. Top, examples of Na+ currents, induced by a series of depolarizing steps shown on top, recorded before and after GDNF application. Bottom, *I–V* curve showing that the peak Na^+ currents were not changed after GDNF application (n = 8). (**d**) Effect of GDNF on Ca^{2+} currents. Cells were recorded in an extracellular solution containing TEA, 4-AP and TTX. Ba^{2+} was used as the charge carrier. Top and middle, examples of LVA and HVA Ca^{2+} currents before and after GDNF application. Voltage protocols are shown on top of current traces. Bottom, *I–V* curves for both LVA and HVA currents. Peak HVA Ca^{2+} currents were increased after GDNF application (n = 12).

with vehicle and 5.6 ms when perfused with GDNF, suggesting an increase in the latency of single channel opening. To further understand how GDNF modulates A-type K^+ channels, we plotted steady-state activation curves[31]. The reversal potential (V_{rev}) of I_A, obtained by analyzing the tail currents elicited by a series of hyperpolarizing voltage steps following a strong depolarization, was approximately –96 mV (**Fig. 3d**). GDNF also elicited a strong inhibitory effect on maximal conductance (g_{max}) calculated at +80 mV depolarization (**Table 1**). Membrane conductances (g) at different test potentials were derived by dividing the peak I_A currents by the current driving force ($V_{test} - V_{rev}$), and were normalized to g_{max} (**Fig. 3e**). Treatment with GDNF produced an 11.4 mV rightward shift in the activation curve ($V_{1/2}$ was 7.3 ± 1.3 mV for control and 18.7 ± 2.1 mV for GDNF-treated cells, respectively, $p < 0.01$). Using long conditional voltage steps (from –100 mV to +35 mV, increments, 800 ms long) followed by a testing step of +50 mV, we obtained inactivation curves. GDNF did not have significant effect on I_A inactivation (**Fig. 3e**). To confirm that the effect of GDNF on neuronal excitability is mediated by I_A, we used 4-AP, the blocker for A channels. Application of 4-AP (10 mM) mimicked the excitatory effect of GDNF; the spike frequency was increased and the first spike latency was reduced (**Fig. 3f**, **Table 2**). Furthermore, application of 4-AP occluded the effect of GDNF on neuronal firing (**Fig. 3f**, **Table 2**). Taken together, these results suggest that GDNF attenuates the activation of A-type K^+ channel, without affecting its inactivation.

Table 2. Effect of 4-AP on the excitabilities of cultured midbrain neurons.

	Control	4-AP	4-AP + GDNF	*n*
First spike latency (ms)	128.8 ± 57.6	39.3 ± 17.8*	35.8 ± 20.2*	6
Spike frequency (Hz)	11.9 ± 3.9	31.1 ± 8.7*	29.0 ± 8.4*	6

First spike latency, the time needed from current injection to first spike appearance. Spike frequency, the inverse of the interval between the first two spikes. Values given are mean ± s.d. Both '4-AP' and '4-AP + GDNF' groups are significantly different from the control group, but there was no difference between '4-AP' and '4-AP + GDNF' groups (ANOVA plus *post hoc* analysis, *$p < 0.01$).

To determine the intracellular signaling mechanisms that mediate the acute effect of GDNF, we measured the activity of MAP kinase in the midbrain neurons. Application of GDNF induced rapid phosphorylation of Erk1 and Erk2, two isoforms of MAP kinase, as detected by western blot using an antibody that specifically recognizes the phosphorylated forms of MAP kinase (**Fig. 4a**). Dose–response experiments indicated that GDNF activated MAP kinase at a concentration as low as 1 ng/ml, but higher concentrations were needed to fully activate the enzyme (**Fig. 4d**). The effect of GDNF on MAP kinase phosphorylation was completely blocked by U0126 (**Fig. 4b**) or PD098059 (PD, **Fig. 4c**), which are specific inhibitors for MEK, an enzyme that phosphorylates MAP kinase. Interestingly, treatment of the cultures with U0126 (**Fig. 4e**) or PD (data not shown) alone also inhibited the basal levels of phosphorylated MAP kinase in these midbrain neurons. One of the potential targets for MAP kinase

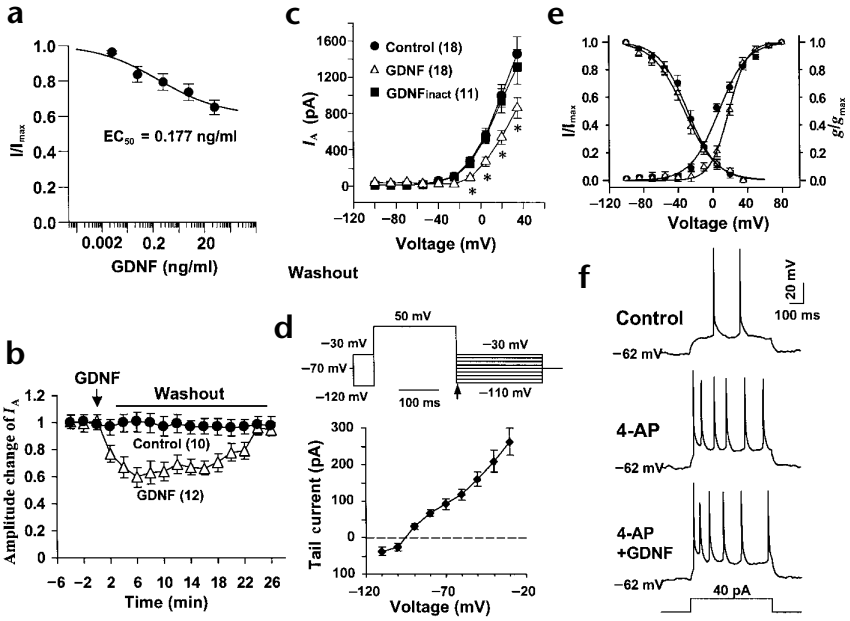

Fig. 3. Pharmacological and kinetic analysis of GDNF modulation of I_A. (**a**) Dose–response curve (n = 16). The peak currents were normalized to the maximum I_A in control conditions. (**b**) Time course. The suppression of I_A by GDNF is reversed after GDNF is washed out. (**c**) I–V curve showing that GDNF shifts the curve towards right. *Significantly lower than control (Student's t-test, $p < 0.05$). Number of cells recorded are in parentheses. (**d**) Reversal potential of I_A. Top, two voltage protocols used to elicit I_A tail currents. The first was initially held at –120 mV, depolarized to 50 mV, and then hyperpolarized by a series of voltage steps from –30 mV to –110 mV (10-mV increments). The second was essentially the same as the first, except that the cell was initially held at –30 mV. The currents generated by the two protocols were subtracted, and the amplitudes of I_A tail currents were measured at the times indicated by the arrow. Bottom, plot of averaged I_A tail currents as a function of hyperpolarizing voltages. Dashed line, 0 current level. (**e**) Steady-state activation and inactivation curves before and after GDNF treatment. For activation curves, I_A currents were generated by the two-voltage protocol similar to that described in Fig. 2b, but the test depolarization potentials (V_{test}) were a series of 15-mV increment steps, from –100 mV to +80 mV. The reversal membrane potential (V_{rev}) under our recording condition was –96 mV. Membrane conductances (g) at different test potentials were obtained by dividing the peak I_A currents by the current driving force ($V_{test} - V_{rev}$), and were normalized to that collected at +80 mV depolarization (g_{max}). The data were fitted with a Boltzmann function, $g/g_{max} = 1/(1 + \exp(-(V - V_{1/2})/k))$. Here, V is the membrane potential, $V_{1/2}$ is the membrane voltage at which the current amplitude is half-maximum, and k is the slope factor (n = 23). For inactivation curves, a long conditional step of various voltages (from –100 mV to +35 mV, increments of 15 mV, 800 ms) was followed by a testing step of +50 mV. The peak current amplitudes were normalized to that collected at –100 mV conditional step (I_{max}), and plotted as a function of the conditional step potentials. The data were fitted with a Boltzmann function, $I/I_{max} = 1/(1 + \exp(-(V_{1/2} - V)/k))$. Here, V is the membrane potential, $V_{1/2}$ is the half-inactivation potential and k is the slope factor. n = 18. (**f**) Occlusion of GDNF effect by the A channel blocker 4-AP. n = 6. Acute application of 4-AP increased the neuronal excitability, and occluded the effect of GDNF on neuronal firing.

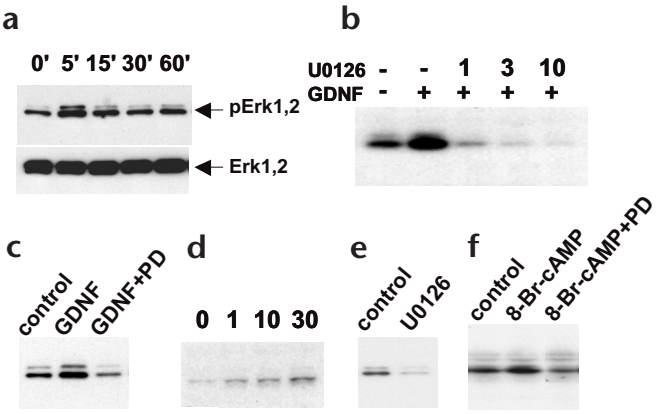

Fig. 4. Activation of MAP kinase by GDNF or other agents in cultured midbrain neurons. GDNF or other agents were applied directly to the culture medium. MAP kinase activation was detected by an antibody that specifically recognizes phosphorylated form of MAP kinase. (**a**) Time course of GDNF-induced MAP kinase phosphorylation. Top, phosphorylated MAP kinase (pErk1, 2) at different time points after GDNF application. Bottom, total MAP kinase (Erk1, 2) in the same blot. (**b**) Dose–response effect of the MEK inhibitor U0126 on GDNF-induced MAP kinase phosphorylation. Cultures were pretreated with U0126 at 0, 1, 3 or 10 μM for 30 min before GDNF application. (**c**) Inhibitor for MAP kinase pathway PD098059 (PD, 10 μM) prevents GDNF-induced MAP kinase phosphorylation. (**d**) Dose-dependent activation of MAP kinase. 0, 1, 10 or 30 ng/ml GDNF. (**e**) Inhibition of endogenous MAP kinase activity by U0126. (**f**) Activation of MAP kinase by bath application of 8-bromo-cAMP (1 mM).

phosphorylation is the A channel[32]. We therefore tested whether inhibition of MAP kinase activity could prevent the GDNF modulation of A-current. Indeed, pretreatment of the midbrain neurons with either PD or U0126 completely prevented the inhibitory effect of GDNF on I_A (**Fig. 5a**, top; **Fig. 5b**). Application of PD or U0126 alone significantly increased the peak amplitude of I_A (**Fig. 5a**, middle; **Fig. 5b**), suggesting that endogenous, tonic levels of MAP kinase activity in these neurons suppress A channels from functioning fully. Moreover, activation of MAP kinase by methods other than GDNF perfusion also suppressed I_A. Application of 8-bromo-cyclic AMP (8-Br-cAMP, 1 mM, **Fig. 4f**) or brain-derived neurotrophic factor (BDNF, 2 nM, data not shown) rapidly activated MAP kinase. The peak amplitude of I_A was significantly reduced when the neurons were perfused with either 8-Br-cAMP or BDNF, and the effect of 8-Br-cAMP was blocked by PD (**Fig. 5a**, bottom; **Fig. 5b**). Finally, GDNF application also increased the neuronal excitability under current clamp, whereas pretreatment with PD prevented the GDNF effect ($n = 7$, **Fig. 5c**). Altogether, these findings strongly suggest that GDNF modulation of A-current and neuronal excitability in the midbrain dopaminergic neurons is mediated by MAP kinase activation.

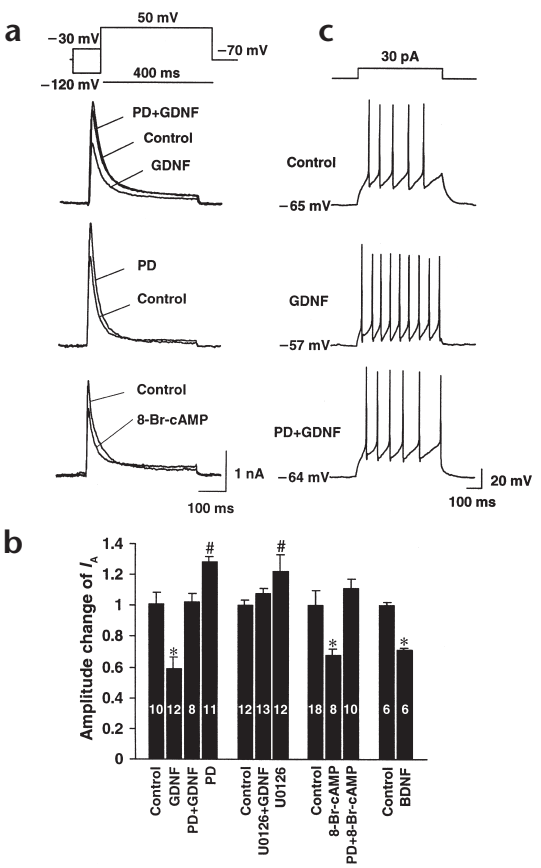

Fig. 5. Role of MAP kinase in GDNF modulation of neuronal excitability and A-currents. (**a**) Voltage-clamp recordings. I_A can be modulated by alteration of MAP kinase activity. Top, PD pretreatment prevented GDNF inhibition of I_A. Middle, inhibition of basal MAP kinase activity by PD increases I_A currents. Bottom, activation of MAP kinase by 8-bromo-cAMP inhibits I_A currents. (**b**) Effects of alteration of MAP kinase activity on the peak amplitude of I_A. Numbers associated with each bar are the number of recordings obtained. *Significantly lower than control. #Significantly higher than control. (ANOVA plus *post hoc* analysis, $p < 0.05$). (**c**) Current-clamp recordings showing that PD blocked the effect of GDNF on excitability.

To investigate whether GDNF modulates the excitability of dopaminergic neurons in the intact adult substantia nigra, we performed intracellular recording of neurons from freshly prepared midbrain slices. Dopaminergic neurons were identified not only by their location in the slices, but also by their characteristic time-dependent inward rectification, or 'sag' potentials in response to a hyperpolarization current injection (**Fig. 6a**)[27,33]. The resting membrane potentials were usually around –55 to –65 mV, and depolarizing current injection into neuronal soma elicited a few action potential spikes (**Fig. 6b**). Because growth factors are usually difficult to penetrate into brain slices, we used twice as high concentration of GDNF (50 ng/ml) to ensure delivery of GDNF into the slices within reasonable amount of time. Similar to that in cultured neurons, application of GDNF rapidly induced approximately 8.0 ± 1.9 mV membrane depolarization (**Fig. 6b**). When the membrane potentials were corrected to the originals by current injection, however, GDNF still increased spike frequency and decreased first spike latency (**Fig. 6b** and **d**). Pretreatment of slices with PD completely prevented the GDNF effects (**Fig. 6c**). Moreover, application of PD alone also reduced spike frequency and first spike latency (**Fig. 6c**), and application of PD after GDNF reversed the excitatory effects of GDNF (**Fig. 6b**). Interestingly, the effect of GDNF on membrane depolarization was neither blocked (data not shown) nor reversed (**Fig. 6b**) by PD. This result is consistent with the effect of GDNF on other channels in addition to A-type K^+

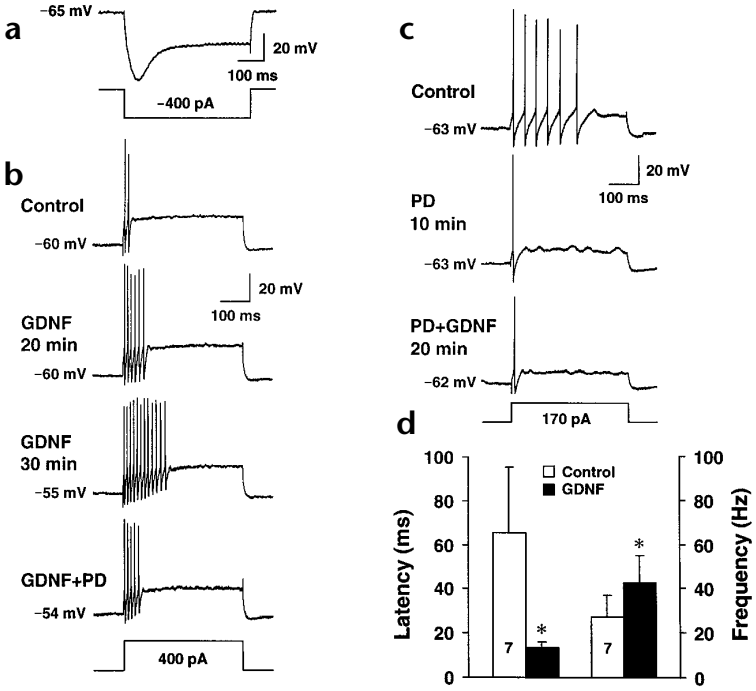

Fig. 6. Effect of GDNF on the excitability of dopaminergic neurons in midbrain slices. (**a**) An example of hyperpolarization-induced 'sag' potential characteristic of midbrain dopaminergic neurons. (**b**) GDNF acutely potentiates neuronal excitability. A depolarizing current (400 pA) was injected into a midbrain neuron, and changes in membrane potentials were recorded under the current-clamp mode. GDNF (50 ng/ml) was perfused into the slice chamber. Application of GDNF resulted in a time-dependent increase in spike frequency, and application of PD after GDNF reversed the excitatory effects. (**c**) The MAP kinase pathway inhibitor PD blocks the effect of GDNF. Application of PD reduced the spike frequency, and application of GDNF after PD no longer elicited the excitatory effect. (**d**) Summary of GDNF effects on spike frequency and first spike latency (n = 7). *Significantly different from control (paired t-test, $p < 0.05$).

channels (such as leakage current I_L or hyperpolarization-activated cation current I_H) through a MAP kinase-independent mechanism. In conclusion, GDNF potentiates neuronal excitability in developing as well as adult dopaminergic neurons in the midbrain.

Discussion

Our observations support three conclusions. First, GDNF rapidly and reversibly inhibits A-type potassium channels in the midbrain dopaminergic neurons. Second, the acute modulation of A-current by GDNF results in a potentiation of the excitability of these neurons. Third, the acute effects of GDNF are mediated through a mechanism that involves activation of MAP kinase. These findings reveal a previously unexpected role of GDNF in the function of normal midbrain dopaminergic neurons.

Acute regulation of ion channels by neurotrophic factors has been a subject of great interest in recent years. BDNF, for example, has been shown to elicit a provocative, rapid effect on ion channels in pontine and hippocampal neurons[34,35]. We show that GDNF also elicited a rapid and reversible inhibition of A-type K^+ channels in the midbrain dopaminergic neurons. This effect was achieved, at least in part, by attenuating the steady-state activation of the A channels. Interestingly, GDNF selectively modulated the large, TH-positive dopaminergic neurons, without affecting the small, non-dopaminergic neurons. Several types of A-type K^+ channels, including Kv1.4, Kv4.2 and Kv4.3, are expressed in these midbrain dopaminergic neurons[36,37]. Inhibition of A-currents clearly contributed to the potentiating effects of GDNF on neuronal excitability, as reflected by the changes in depolarization-induced repetitive firing of action potentials. GDNF modulation of A-type potassium channels led to a reduction in the first spike latency, an increase in spike frequency and a removal of the notch-like repolarization[25,28–30].

In addition to its effect on A-current, GDNF consistently elicited a small depolarization. In midbrain slices, GDNF elicited a 33% decrease (measured by hyperpolarizing steps), rather than an increase, in input resistance. We investigated whether GDNF regulation of other ion channels contributes to its effect on neuronal excitability. GDNF had no effect on Na^+ channels. Most of our recordings were done in the presence of Cd^{2+} to block Ca^{2+} channels. The decrease in input resistance could not have been due to an increase in I_L, as this would result in hyperpolarization, not depolarization. Thus, the decrease in input resistance was most likely caused by GDNF modulation of I_H, resulting in a depolarization at resting membrane potential. However, the contribution of I_H to GDNF effects on neuronal excitability should be quite small for the following reasons. First, the 33% decrease in input resistance could not account for the 80% decrease in the first spike latency. Second, changes in I_H should not affect the spike frequency of repetitive firings evoked by step depolarization. Finally, the reversal potential for I_H is approximately –40 mV[38], and I_H has a very characteristic anomalous rectification property[39]. A step depolarization, therefore, should rapidly and dramatically decrease I_H. Thus, even if there were changes in I_H, they should have minimal influences on the modulation of spike frequency and first spike latency induced by GDNF. Taken together, the increase in the excitability of dopaminergic neurons in midbrain slice is most likely due to the GDNF modulation of A-type K^+ channels.

Although BDNF is known to acutely regulate ion channels[34,35], mechanisms underlying such regulation remain largely unknown. Here we provided several lines of evidence suggesting that the acute effects of GDNF on A-current and neuronal excitability are mediated by MAP kinase signaling pathway. First, GDNF rapidly and dose-dependently activated MAP kinase in cultured neurons derived from ventral midbrain. Second , GDNF-induced effects on A-current and neuronal excitability were blocked by specific inhibitors for the MAP kinase pathway. Third, inhibition of the endogenous MAP kinase in the absence of GDNF increased A-current. Fourth, activation of MAP kinase by 8-Br-cAMP or BDNF also inhibited A-current. Thus, MAP kinase seems to be a key converging point for the acute regulation of A-type K^+ channels. MAP kinase phosphorylates the A-type K^+ channel Kv4.2 on specific Ser/Thr residues in the C-terminal cytoplasmic domain[32]. Moreover, activation of MAP kinase seems to suppress A-type K^+ channel activity on the distal dendrites of hippocampal pyramidal neurons (L.L. Yuan *et al., Soc. Neurosci. Abstr.* **26**, 520.6, 2000). We have now identified an endogenous factor that inhibits A-type K^+ channels selectively in the midbrain dopaminergic neurons, through MAP kinase activation.

The present study reveals an unexpected unexpected function of GDNF in the midbrain dopaminergic neurons, which project their axons to striatum and other brain areas. These neurons are involved in the regulation of motor function as well as

cognitive processes, and are the major population degenerated in Parkinson's disease. Substantial evidence supports the notion that GDNF prevents and reverses the degeneration of the midbrain dopaminergic neurons induced by many chemical and mechanical insults[10–15]. However, homozygous mice lacking genes for GDNF or its receptor GFRα1 do not exhibit any loss in the number of TH-positive dopaminergic neurons in the nigra, or impairments in the dopaminergic projections to the striatum[40–42] (but see ref. 43). Injection of GDNF into the nigra or striatum of normal rats does not increase the number of nigra dopaminergic neurons[44,45]. These results suggest that although it is a potent neurotrophic factor for injured or dying dopaminergic neurons, the major function of GDNF in normal midbrain may not be (or is in addition to) the regulation of the long-term survival of dopaminergic neurons. Interestingly, a single injection of GDNF to nigra of normal rats elicits a dose-dependent increase in dopamine release in the striatum, as well as an enhancement of locomotor activity[46,47]. We now show in both cultured neurons and in slices that GDNF acutely potentiates the excitability of midbrain dopaminergic neurons. It is conceivable that the GDNF-induced potentiation of neuronal excitability could result in an increase in dopamine release, leading to an enhancement in locomotor activity. Thus, our findings suggest an acute role of GDNF in regulating the function of midbrain dopaminergic neurons. These results may also have implications in the treatment of Parkinson's disease.

Methods

Culture preparation. All experiments involving animals were conducted according to NIH animal use guidelines. Ventral part of midbrain was dissected from embryonic day 14 (E14) Sprague–Dawley rats, dissociated in Ca^{2+} and Mg^{2+}-free Hanks balanced salt solution containing 0.125% trypsin for 30 min, and plated at 0.3 million cells per 35 mm dish. The cells were grown at 37°C, 5% CO_2 and 95% humidity, first in 10% FBS/DMEM, and switched one day later to serum-free Neurobasal medium containing B27 supplement (Life Technologies, Rockville, Maryland). The cultures were grown for more than 10 days and media were changed every other day.

Electrophysiological recordings. Action potentials from cultured midbrain neurons were recorded using standard, whole-cell current clamp techniques[48]. During experiments, culture dishes were rinsed twice and continuously perfused with extracellular (Ringer's) solution containing 140 mM Na isethionate, 3 mM K gluconate, 1 mM Na pyruvate, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM dextrose, and 10 mM HEPES, pH 7.3, 320 mOsm. Cd^{2+} (0.4 mM) was added to the Ringer's solution to block all Ca^{2+} channels and Ca^{2+}-activated K^+ channels. Recordings of ion channels were performed under voltage clamp conditions[31]. For A-current, the extracellular Ringer's solution was supplemented with 1 μM TTX and 0.4 mM $CdCl_2$ to block Na^+ and Ca^{2+} channels, respectively, and TEA (5 mM) to block delayed rectified potassium channels. For Na^+ currents, the same Ringer's solution was used, except TTX was replaced with 4-AP (5 mM). The intracellular pipette solution used for action potential, A-current and Na^+ current recordings contained 130 mM K-gluconate, 10 mM HEPES, 11 mM EGTA, 2 mM $MgCl_2$, 10 mM phosphocreatine, 2 mM Mg-ATP and 0.5 mM Na_2-GTP, pH 7.3. Ca^{2+} currents were recorded essentially as described[49]. The extracellular solution contained 50 mM NaCl, 5 mM $BaCl_2$, 90 mM TEA, 5 mM 4-AP, 0.001 mM TTX and 10 mM HEPES, pH 7.3. The intracellular pipette solution contained 122 mM CsCl, 9 mM HEPES, 4.5 mM EGTA, 2.2 mM $MgCl_2$, 14 mM phosphocreatine, 4 mM Mg-ATP and 1 mM Na_2-GTP, pH 7.3. Whole-cell electrodes were 2–4 MΩ in resistance. All recordings were done in room temperature. Data were collected by a patch clamp amplifier (Axopatch 200A), stored in a PC, and analyzed by p-Clamp software (Axon Instruments, Foster City, California). After whole-cell configuration was achieved, series resistance were compensated by 80–90% and monitored periodically. Most cells had series resistance around 7–8 MΩ (range, 4–13 MΩ). A small percentage of cells with a resting membrane potential less than –50 mV, or gradual changes in membrane potential, input resistance or action potential amplitudes, were considered as unhealthy and discarded. For current-clamp recordings, a depolarizing current step was injected to induce multiple action potentials. For voltage-clamp recordings, voltage protocols given by p-clamp were delivered to the cells through the patch pipette. We routinely monitored the cell 5–10 min before application of drugs. GDNF was applied through gravity perfusion using multi-barrel perfusion pipette positioned approximately 100 μm to the cell under recording.

For intracellular recording from brain slices, horizontal slices of midbrain (300 μm thick) were prepared as previously described[27,33] using 150–200 g male Sprague–Dawley rats. Recordings were made from submerged slices in a recording chamber (0.5 ml) perfused at a rate of 1.5–2.0 ml/min with oxygenated (95% O_2/5% CO_2) solution at 35°C containing 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 18 mM $NaHCO_3$ and 11 mM glucose. Current-clamp recordings with KCl (2M)-filled electrodes (tip resistance, 40–100 MΩ) were made with the aid of a dissection microscope. Potentials were amplified with an Axoclamp 2A amplifier and recorded using Axotape. Dopaminergic neurons in midbrain were identified by their characteristic electrophysiological and pharmacological properties[33]. Before application of GDNF (50 ng/ml, diluted in 60 μg/ml BSA), $CdCl_2$ was added to the perfusate at the final concentration of 0.4 mM to block after hyperpolarization.

Labeling of GDNF responsive cells. The cells tested to be responsive to GDNF were loaded with Alexa Fluor 594 (Molecular Probes, Eugene, Oregon) as a marker, and the cultures were washed with phosphate buffer saline (PBS, pH 7.4), fixed with 2% paraformaldehyde for 20 min, and washed twice with PBS. Fixed neurons were permeabilized with PBS containing 0.1% Triton X-100 for 1 h. After incubation in the blocking solution (3% normal goat serum and 3% bovine serum albumin in PBS) for 2 h, cells were incubated with anti-tyrosine hydroxylase (TH, 1: 500, Chemicon, Temecula, California) antibody at 4°C for overnight. The TH-positive cells were visualized using FITC-goat anti-rabbit IgG antibody (1:200, green), while the GDNF responsive cells were labeled by Alexa Fluor 594 (red). The fluorescence images were captured by a MicroMax CCD camera (Roper Scientific, Trenton, New Jersey), and processed by IPLab software (Scanalytics, Fairfax, Virginia).

Western blot analysis. MAP kinase activation was determined by western blot as described[26]. Midbrain cultures were stimulated with GDNF or other agents for the indicated times. The stimulation was terminated by addition to the cultures of 0.1 ml of lysis buffer. Cells were harvested and insoluble materials were removed by centrifugation. Equal amounts of proteins were separated in a 12% SDS-acrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, Massachusetts). The membranes were probed with an anti-active MAP kinase antibody (1: 1000, Promega, Madison, Wisconsin), which recognizes phosphorylated MAP kinase (Erk1-P and Erk2-P). MAP kinase activation was detected by a chemiluminescence method (Pierce, Rockford, Illinois). The antibody was then stripped and reprobed with an anti- MAP kinase antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, California) to ensure that equal amount of MAP kinase (Erk1 and Erk2) proteins were loaded in all lanes.

Acknowledgements

We thank C. McBain, J.-f. Zhang and J. Yang for discussions and comments on the manuscript. This work is supported in part by a grant from Shanghai Science and Technology Commission.

Received 5 July; Accepted 5 September 2001

1. Airaksinen, M. S., Titievsky, A. & Saarma, M. GDNF family neurotrophic factor signaling: four masters, one servant? *Mol. Cell. Neurosci.* **13**, 313–325 (1999).
2. Baloh, R. H., Enomoto, H., Johnson, E. M. Jr. & Milbrandt, J. The GDNF family ligands and receptors—implications for neural development. *Curr. Opin. Neurobiol.* **10**, 103–110 (2000).

3. Durbec, P. *et al.* GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789–793 (1996).
4. Jing, S. *et al.* GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85**, 1113–1124 (1996).
5. Treanor, J. J. *et al.* Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80–83 (1996).
6. Trupp, M. *et al.* Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* **381**, 785–788 (1996).
7. Feng, L. *et al.* Differential signaling of glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor in cultured ventral mesencephalic neurons. *Neuroscience* **93**, 265–273 (1999).
8. Soler, R. M. *et al.* Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. *J. Neurosci.* **19**, 9160–9169 (1999).
9. Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. & Collins, F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–1132 (1993).
10. Tomac, A. *et al.* Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* **373**, 335–339 (1995).
11. Gash, D. M. *et al.* Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* **380**, 252–255 (1996).
12. Hoffer, B. J. *et al.* Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons *in vivo*. *Neurosci. Lett.* **182**, 107–111 (1994).
13. Sauer, H., Rosenblad, C. & Bjorklund, A. Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proc. Natl. Acad. Sci. USA* **92**, 8935–8939 (1995).
14. Cass, W. A. GDNF selectively protects dopamine neurons over serotonin neurons against the neurotoxic effects of methamphetamine. *J. Neurosci.* **16**, 8132–8139 (1996).
15. Beck, K. D. *et al.* Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* **373**, 339–341 (1995).
16. Lindsay, R. M., Altar, C. A., Cedarbaum, J. M., Hyman, C. & Wiegand, S. J. The therapeutic potential of neurotrophic factors in the treatment of Parkinson's disease. *Exp. Neurol.* **124**, 103–118 (1993).
17. Gao, W., Dugich-Djordjevic, M. M., Weil, R. J. & Lu, B. Therapeutical usage of neurotrophic factors: patent analysis. *Expert Opin. Therap. Patents* **7**, 325–338 (1997).
18. Lu, B. & Chow, A. Neurotrophins and hippocampal synaptic plasticity. *J. Neurosci. Res.* **58**, 76–87 (1999).
19. McAllister, A. M., Katz, L. C. & Lo, D. C. Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci.* **22**, 295–318 (1999).
20. Mount, H. T., Dean, D. O., Alberch, J., Dreyfus, C. F. & Black, I. B. Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. *Proc. Natl. Acad. Sci. USA* **92**, 9092–9096 (1995).
21. Trupp, M. *et al.* Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian perioheral neurons. *J. Cell Biol.* **130**, 137–148 (1995).
22. Oppenheim, R. W. *et al.* Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: regulation of programmed cell death among motoneuron subtypes. *J. Neurosci.* **20**, 5001–5011 (2000).
23. Nguyen, Q. T., Parsadanian, A. S., Snider, W. D. & Lichtman, J. W. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science* **279**, 1725–1729 (1998).
24. Wang, C. *et al.* Ca^{2+}-binding protein frequenin mediates GDNF-induced synaptic facilitation by potentiating Ca^{2+} channels. *Neuron* (in press).
25. Jahnsen, H. & Llinas, R. Ionic basis for the electro-responsiveness and oscillatory properties of guinea-pig thalamic neurones *in vitro*. *J. Physiol. (Lond.)* **349**, 227–247 (1984).
26. Feng, L. *et al.* Differential effects of GDNF and BDNF on cultured ventral mesencephalic neurons. *Mol. Brain Res.* **66**, 62–70 (1999).
27. Lacey, M. G., Mercuri, N. B. & North, R. A. Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *J. Neurosci.* **9**, 1233–1241 (1989).
28. Connor, J. A. & Stevens, C. F. Prediction of repetitive firing behaviour from voltage clamp data on an isolated neurone soma. *J. Physiol. (Lond.)* **213**, 31–53 (1971).
29. Llinas, R. The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. *Science* **242**, 1654–1664 (1988).
30. McCormick, D. A. & Huguenard, J. R. A model of the electrophysiological properties of thalamocortical relay neurons. *J. Neurophysiol.* **68**, 1384–400 (1992).
31. Zhang, L. & McBain, C. J. Potassium conductances underlying repolarization and after-hyperpolarization in rat CA1 hippocampal interneurones. *J. Physiol. (Lond.)* **488**, 661–672 (1995).
32. Adams, J. P. *et al.* The A-type potassium channel kv4.2 is a substrate for the mitogen-activated protein kinase ERK. *J. Neurochem.* **75**, 2277–2287 (2000).
33. Johnson, S. W. & North, R. A. Two types of neurons in the rat ventral tegmental area and their synaptic inputs. *J. Physiol. (Lond.)* **450**, 455–468 (1992).
34. Kafitz, K. W., Rose, C. R., Thoenen, H. & Konnerth, A. Neurotrophin-evoked rapid excitation through TrkB receptors. *Nature* **401**, 918–921 (1999).
35. Li, H. S., Xu, X. Z. & Montell, C. Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. *Neuron* **24**, 261–273 (1999).
36. Serodio, P. & Rudy, B. Differential expression of Kv4 K^+ channel subunits mediating subthreshold transient K^+ (A-type) currents in rat brain. *J. Neurophysiol.* **79**, 1081–1091 (1998).
37. Chung, Y. H., Shin, C., Kim, M. J. & Cha, C. I. Immunohistochemical study on the distribution of six members of the Kv1 channel subunits in the rat basal ganglia. *Brain Res.* **875**, 164–170 (2000).
38. Washio, H., Takigachi-Hayashi, K. & Konishi, S. Early postnatal development of substantia nigra neurons in rat midbrain slices: hyperpolarization-activated inward current and dopamine-activated current. *Neurosci. Res.* **34**, 91–101 (1999).
39. Mercuri, N. B., Bonci, A., Calabresi, P., Stefani, A. & Bernardi, G. Properties of the hyperpolarization-activated cation current Ih in rat midbrain dopaminergic neurons. *Eur. J. Neurosci.* **7**, 462–469 (1995).
40. Moore, M. W. *et al.* Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76–79 (1996).
41. Sanchez, M. P. *et al.* Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70–73 (1996).
42. Cacalano, G. *et al.* GFRα1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21**, 53–62 (1998).
43. Granholm, A. C. *et al.* Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J. Neurosci.* **20**, 3182–3190 (2000).
44. Bowenkamp, K. E. *et al.* Glial cell line-derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. *J. Comp. Neurol.* **355**, 479–489 (1995).
45. Beck, K. D. *et al.* GDNF Induces a dystonia-like state in neonatal rats and stimulates dopamine and serotonin synthesis. *Neuron* **16**, 665–673 (1996).
46. Hudson, J. *et al.* Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits *in vivo*. *Brain Res. Bull.* **36**, 425–432 (1995).
47. Herbert, M. A., Horne, C. G. V., Hoffer, B. J. & Gerhardt, G. A. Functional effects of GDNF in normal rat striatum: presynaptic studies using *in vivo* electrochemistry and microdialysis. *J. Pharmcol. Exp. Ther.* **279**, 1181–1190 (1996).
48. Kim, H. G., Wang, T., Olafsson, P. & Lu, B. Neurotrophin 3 potentiates neuronal activity and inhibits g-aminobutyratergic synaptic transmission in cortical neurons. *Proc. Natl. Acad. Sci. USA* **91**, 12341–12345 (1994).
49. Plant, T. D., Schirra, C., Katz, E., Uchitel, O. D. & Konnerth, A. Single-cell RT-PCR and functional characterization of Ca^{2+} channels in motoneurons of the rat facial nucleus. *J. Neurosci.* **18**, 9573–9584 (1998).
50. Anderson, P., Storm, J. & Wheal, H. V. Thresholds of action potentials evoked by synapses on the dendrites of pyramidal cells in the rat hippocampus in *vitro*. *J. Physiol. (Lond.)* **383**, 509–526 (1987).