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Comparison of Modulation of Kv1.3 Channel by Two Receptor Tyrosine Kinases in Olfactory Bulb Neurons of Rodents

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Abstract

Activation of the receptor tyrosine kinase (RTK), insulin (IRK), or neurotrophin B (TrkB) was characterized and compared in olfactory bulb neuron (OBN) cultures from Sprague Dawley rats and sv129 B6 mice. Current suppression attributed to modulation of the delayed rectifier, Kv1.3, a voltage-gated potassium (Kv) channel of the *Shaker* family, was observed following acute application of the growth factors, insulin, or brain-derived neurotrophic factor (BDNF), to mitral cells of either rodent model. Using site-directed mutagenesis of putative tyrosine phosphorylation recognition motifs in the channel, we find that stimulation of Kv1.3 with these growth factors causes multiple phosphorylation, albeit via different residue combinations that are RTK specific.

Keywords

BDNF; Insulin; Neurotrophin; Olfactory; TrkB; Tyrosine Phosphorylation

Introduction

The activity of ion channels is dynamic, responding to intercellular signaling molecules and cytoplasmic factors via allosteric interactions and covalent modifications (Kaczmarek and Levitan 1987; Levitan 1994; Pawson and Scott 1997). To understand the capacity of electrical signaling, one must ultimately elucidate the mechanisms by which channel proteins respond to biochemical changes at specific modulatory sites (Levitan 1994, 1999). One such modulation occurs via phosphorylation. Phosphorylation of tyrosine residues, as a result of intercellular communication, modulates enzymatic activity and creates binding sites for the recruitment of downstream signaling proteins (Manning et al. 2002; Hubbard and Till 2000). To date, several different types of tyrosine kinases have been demonstrated to produce short-term modulatory changes in neuronal excitability or ion channel function (Huang et al. 1993; Wang and Salter 1994; Holmes et al. 1996b; Jonas and Kaczmarek 1996; Bowlby et al. 1997; Sherwood et al. 1997; Yu et al. 1997; Fadool and Levitan 1998; Berninger and Poo 1999; Rogalski et al. 2000).

The olfactory bulb (OB) represents a unique division of the central nervous system where a continual regeneration of basal cells produces neurogenesis in the adult. Growth factors, neurotrophins, and their receptors are developmentally expressed postnatally but then persist in the adult OB at relatively high levels (Baskin 1983; Hill et al. 1986; Gupta et al. 1992; Fadool et al. 2000; Tucker and Fadool, 2002). Thus it could be postulated that the growth

factors have a dual role in this system. They act in their traditional role as trophic factors during the growth and development of the olfactory system, but as members of receptor tyrosine kinase signaling cascades, they persist in the adult to modulate electrical activity via phosphorylation of ion channel proteins. Olfactory receptor neurons contained in the nasal epithelium must be regenerated approximately every thirty days (Graziadei and Monti-Graziadei 1978) and modulation of electrical activity in the target OB could mediate circuit development, axonal pathfinding, or proper survivability and regeneration of these neurons.

The delayed rectifier, Kv1.3, a voltage-gated potassium (Kv) channel of the *Shaker* family, carries 60–80% of the K⁺ current in rat OB neurons and has been found to be a molecular target for multiple phosphorylation by several tyrosine kinases including growth factors (Holmes et al. 1996a; Bowlby et al. 1997; Fadool et al. 1997; Fadool and Levitan 1998; Fadool et al. 2000). Kv channels in the OB partly determine the membrane potential of a cell, regulate the level of neuronal excitability by influencing the duration of the action potential, determine the frequency of repetitive firing, and time the interspike interval (Jan and Jan 1994). Long-term treatment of individuals with metabolic disorders or nerve injury demands an understanding of the modulatory events that may occur at the level of the ion channel to alter electrical signaling in individuals taking activators of tyrosine kinase receptors (diabetics, spinal injury patients).

Herein we characterize the suppression of the predominant Kv current in native OB neurons from the rat and mouse, by two different receptor tyrosine kinases (RTKs), insulin (IR) and neurotrophin B (TrkB) receptor kinase. The goal of the study was to compare the mechanism of modulation of this K channel by two RTKs that have different modes of activation and downstream signaling pathways. Biophysical properties of Kv1.3 currents in mitral cells were characterized and compared across the two rodent models, including peak current amplitude, kinetics of inactivation and deactivation, voltage at half activation, and conductance. Additionally, using a heterologous expression system, BDNF stimulation of wildtype Kv1.3 channel and phosphorylation site mutant channels were studied to determine the molecular target for BDNF-induced tyrosine phosphorylation of the channel.

Results

Comparison of Potassium Channel Properties Across Rat and Mouse in the Olfactory Bulb

Whole-Cell Current Properties—Mouse was selected as the model to study the modulation of native Kv1.3 current properties due to the future perceived utility for studying transgenic animals. Since we could find no reports describing electrophysiological properties of mouse OB neurons in primary culture as opposed to many describing that in rat (i.e., Trombley and Westbrook 1991; Trombley and Shepherd 1993), we initiated our study with a comparison between these two species. Cultured rat and mouse OB neurons were voltage-clamped in the whole-cell configuration. Neurons were held (V_h) at -90 mV and a family of current-voltage responses were generated by stepping the voltage (V_c) from -80 to $+40$ mV in 10 mV increments. The interpulse stimulus interval was at least 30 seconds to prevent cumulative inactivation of Kv1.3 current (Marom et al. 1993; Kupper et al. 1995). A representative family of current-voltage responses is shown in Figure 1A for each species. The plotted current-voltage relationship for a population of neurons demonstrates that voltage-gated properties of outward potassium currents are not inherently different between the two species (Figure 1B). This is best visualized by conversion of the voltage-activated currents to conductance to measure the corresponding voltage at half-activation ($V_{1/2}$) for each species (Figure 1C). As demonstrated in this figure and reported in Table 1, both the $V_{1/2}$ and the slope of the voltage-dependence (κ) were not significantly different between rat and mouse OB neurons (Student's *t*-test, $\alpha \neq 0.05$).

Peak Current Magnitude and Kinetics of Inactivation Over Days In Vitro (DIV)

—Previous confocal imaging of Kv1.3 channel protein expression in rat OB neurons has demonstrated detectable channel protein as early as *DIV*2, which progressively and linearly increases over the first ten days in vitro (Fadool et al. 2000). Thus peak current magnitude and kinetics of inactivation were compared across rat and mouse from *DIV*2-5. Neurons were held (V_h) at -90 mV and depolarized in a single step (V_c) from -80 to $+40$ mV with an interpulse interval of 1 minute. The inactivation time constant (τ_{inact}) did not vary over *DIV*, nor was there a significant difference in the kinetics of inactivation between species (Table 1). As predicted, the peak current magnitude of the outward whole-cell current increased over *DIV*, presumably in register with an increase in Kv1.3 channel protein expression during growth of the neuron in vitro. The peak current magnitude for rat was nearly twice that found in mouse by *DIV*5 (Figure 1D). Given the restricted expression of Kv1.3 on the soma and dendrite of neurons and the notable size differences between rat and mouse OB neurons, it is not unexpected that the total current magnitude was greater in rat than in mouse. The current density values, however, calculated at *DIV*5 for each species were not significantly different (0.14 ± 0.02 pA/pF ($n = 8$) rat versus 0.15 ± 0.03 pA/pF ($n = 11$) mouse) suggesting that the two were congruent in voltage-evoked current normalized to cell size.

Modulation of Mouse Olfactory Bulb Neurons by Activation of Receptor Tyrosine Kinases

—Cultured mouse OB neurons were voltage-clamped in the whole-cell configuration with a V_h of -90 mV and voltage stepped (V_c) from -80 to $+40$ mV in 10 mV increments for a duration of 400 msec using an interpulse interval of 30 seconds. A family of current-voltage relations were generated under control conditions (pretreatment) and 15 minutes following bath application of 50 ng/ml BDNF or 10 μ g/ml insulin. Representative current recordings prior and following neurotrophin or hormone application are found in Figures 2 and 3, respectively. Activation of the endogenous TrkB receptor kinase in these neurons (Deckner et al. 1993; Tucker and Fadool 2002) by the preferred ligand BDNF caused a significant decrease in peak current magnitude (810 ± 149 pA control, 325 ± 119 pA treated, paired t -test, $\alpha < 0.05$, $n = 5$). Similar activation of the endogenous insulin receptor kinase in these neurons (Gupta et al. 1992; Folli et al. 1994; Fadool et al. 2000) by insulin also caused a statistically significant decrease in peak current magnitude (1672 ± 237 pA control, 1227 ± 204 pA treated, paired t -test, $\alpha < 0.05$, $n = 9$). Tyrosine kinase-induced current suppression did not appear to be attributed to a change in voltage dependence of the Kv1.3 channel as indicated by a lack of shift in the current-voltage relation following application of BDNF or insulin (Figures 2 and 3, parts B). The proportional magnitude of current suppression by insulin in rat and mouse OB neurons was qualitatively the same and that by BDNF was roughly twice as great in mouse over that found in rat (Table 1). The capacity for BDNF- or insulin-induced current suppression of OB neurons was independent of *DIV* or initial peak current magnitude.

Modulation of Tyrosine Kinase-Induced Current Suppression is Inhibited by Margatoxin Pretreatment (MgTx)

—Even though the patch/bath solutions in our experiments were designed to favor Kv1.3 current expression in the OB neurons and reduced contribution by other potassium channel species such as calcium- or sodium-activated potassium channels, OB neurons do express two other members of the *Shaker* subfamily, namely that of Kv1.4 and Kv1.5. Thus it was important to ascertain whether receptor tyrosine kinases were acting upon Kv1.3 or other channels potentially contributing to potassium channel conductances. Neurons were held (V_h) at -90 mV and depolarized in a single step (V_c) from -80 to $+40$ mV for a duration of 400 msec with an interpulse interval of 1 minute. After permitting the neuron to stabilize for 3–5 minutes, the scorpion toxin margatoxin (MgTx) was bath applied to a final concentration of 100 pM. At this

concentration, MgTx is selective as a blocker of Kv1.3 channel over that of other *Shaker* family members, and acts with a slow on rate of 10 minutes by blocking the lumen of the channel (Garcia-Calvo et al. 1993; Knaus et al. 1995). Cells were thus pretreated with MgTx and current was monitored for 10–20 minutes, or until further current suppression was not visible. Then insulin or BDNF was applied as previously to test whether either was effective in modulating the remaining non- Kv1.3 potassium current. As shown in Figure 4, pretreated MgTx neurons did not respond to either insulin or BDNF treatment.

Phosphorylation of Kv1.3 Channel Protein by BDNF-Activation of TrkB Kinase

—We have previously demonstrated that acute application of BDNF to adult rat OB causes an increase in tyrosine specific phosphorylation of native Kv1.3 (Tucker and Fadool 2002). A more robust demonstration of the molecular target of activated TrkB kinase can be performed by elimination of phosphorylation and current suppression following mutagenesis of the channel. Therefore, human embryonic kidney (HEK 293) cells were transiently transfected with the cDNA coding for TrkB kinase plus wildtype Kv1.3 or mutant Kv1.3 channel whereby specific tyrosine residues were altered by single point mutagenesis to phenylalanine at four strong recognition motifs for tyrosine specific phosphorylation (Figure 5). Thirty-six hours post transfection, cells were treated for eight minutes with 100 ng/ml BDNF or control vehicle as described in the methodology. Kv1.3 channel proteins were immunoprecipitated with the Kv1.3 specific antiserum, α AU-13, separated by SDS-PAGE, electro-transferred to nitrocellulose and then probed with antiphosphotyrosine (anti-pY) specific antibody, α 4G10, to determine any increase in tyr specific phosphorylation of the wildtype or mutant channel when acutely stimulated with BDNF. Representative immunoblots are shown in Figure 5A. Quantitative immunodensity calculations of 6–7 immunoprecipitations indicate that BDNF treatment causes a 4.2 ± 1.5 fold increase in wildtype channel phosphorylation (Figure 5B). This phosphorylation is lost when Y is changed to F at positions 111–113, 137, or 449 in the carboxyl or amino terminus of the channel. Y479F Kv1.3, however, retains a reduced ability to become phosphorylated by BDNF-activation of TrkB, indicating that this residue is not as important a target for BDNF-induced current suppression of Kv1.3 (Figure 5B). This site may be removed, and still retain a degree of phosphorylation and intermediate current suppression (see also Figure 7 below). Secondly we noted that there is a significant increase in Kv1.3 channel expression when co-transfected with TrkB kinase even in the absence of BDNF (Figure 5C). Quantitative immunodensity calculations for this phosphorylation independent upregulation of the channel are shown in Figure 5D.

Lastly, loss of BDNF-induced Kv1.3 current suppression was sought for YYY111-113FFF, Y137F, and Y449F Kv1.3; all constructs that demonstrated loss of tyrosine phosphorylation by SDS-PAGE analysis under TrkB plus channel cotransfected conditions (Figure 6). HEK 293 cells were transiently transfected as described above but at cDNA concentrations appropriate for physiology (see Methods). Thirty-six hours post transfection, cells containing expressed wildtype and mutant Kv1.3 protein were screened for acute modulation of current by BDNF. Patch pipettes were tip-filled with control patch recording solution and then back-filled with 50 ng BDNF in order to access the TrkB receptor extracellular binding domain. Macroscopic currents were recorded from the cells by voltage-clamping in the cell-attached configuration. Patches were held at -90 mV (V_h) and stepped to a single depolarizing voltage of $+40$ mV (V_c) for a duration of 1000 msec with an interpulse interval of 45 seconds. Figure 6B demonstrates the first recorded trace following stabilization of the patch (Control) and that recorded 20 minutes after achieving the cell-attached configuration (BDNF) for a cell transfected with TrkB plus wildtype Kv1.3 (Wildtype Kv1.3). BDNF significantly reduced the peak magnitude of the wildtype Kv1.3 macroscopic currents without alterations in the kinetics of inactivation or deactivation (Paired *t*-test, α # 0.05, $n = 11$) (Table 2). Figure 6C–F demonstrates similar representative

recordings under the same experimental paradigm for cells co-transfected with TrkB + one of the Kv1.3 channel mutants. BDNF was ineffective in causing significant current suppression for any of the mutant Kv1.3 channels co-expressed with TrkB (Paired *t*-test, α # 0.05) (Table 2) indicating that disruption in any one of the Tyr residues altered the collective phosphorylation sites to disrupt Kv1.3 modulation (Figure 7). Data presented in the combined Figures 5 and 6 provide complementary evidence that multiple residues (Tyr^{111–113}, Tyr¹³⁷, and Tyr⁴⁴⁹) are the molecular target for BDNF modulation of Kv1.3 via phosphorylation by TrkB kinase. Although not statistically significant, but perhaps representing site-specific functions, we noted that deletion of Y137 caused a slight change in the second *Tau* inactivation under BDNF-stimulated conditions (Figure 6D). Likewise deletion of Y449 caused a slight increase in current magnitude under BDNF-stimulated conditions (Figures 6E and 7).

Discussion

The activity of receptor tyrosine kinases (RTKs) is regulated during development of the nervous system. During pruning of neural circuits, during periods of injury, apoptosis, or regeneration, and in disease states, it can become uncoupled from normal regulatory processes or expression levels. We show that activation of two different RTKs, insulin receptor (IR) kinase and neurotrophin receptor B (TrkB), which persist in the adult OB, each cause suppression of a voltage-gated potassium channel, Kv1.3. A direct route for demonstrating the site and specificity of the tyr phosphorylation of Kv1.3 by RTKs is through mutagenesis. In the case of activation of IR kinase, channel modulation was attributed to direct tyrosine phosphorylation of key residues (Y111–113, Y137, and Y479) in the amino and carboxyl terminal of the protein (Fadool et al. 2000). We now show that activation of TrkB kinase by its preferred ligand BDNF similarly modulates Kv1.3 by causing current suppression but utilizes a different combination of multiple tyrosine residues (Y111–113, Y137, and Y449). It is interesting that both these RTKs appear to downregulate the activity of Kv1.3 via multiple phosphorylation given the inherent differences in their activation - IR kinase by binding insulin versus the dimerization of TrkB. Thus, this mechanistic difference in receptor activation does not appear to play a role in the modulation of the potassium channel biophysics.

One possible interpretation for the role of multiple but differential phosphorylation of the channel by BDNF versus insulin, is that downstream signaling molecules of these RTKs may preferentially dock or become activated only when a specific combination of channel residues is phosphorylated. Both of the RTKs become phosphorylated at multiple Y residues in the cytoplasmic portions of the molecules, respectively, which then serve as specific recognition sites for discreet protein-protein interactions with, for example, SH2 containing adaptor proteins (review: Huang and Reichardt 2003; i.e., Ishihara et al. 2003). It would not be surprising if the multiple Y sites along the channel acted similarly to recruit different scaffolding proteins that were specific to activation by insulin or BDNF. Such a phosphorylation-specific interaction has been reported for another Kv family member where Kv1.2 was associated with an actin cytoskeleton-binding protein (cortactin) and this interaction modulated channel function (Hattan et al. 2002). An alternative possibility is that the total quantity of phosphorylation may be a more weighty factor than absolute position of the phosphorylation in encoding the type of modulation for Kv1.3. Activation of the epidermal growth factor, another member of the receptor tyrosine kinase family, does not cause current suppression of Kv1.3 but rather a speeding of the kinetics of channel inactivation. Interestingly, this modulation is attributable to a single phosphorylation (Y479) in the carboxyl terminus and not multiple phosphorylation (Bowlby et al. 1997).

Although both RTKs have the capacity to modulate the channel in a similar manner using an apparently similar molecular mechanism, we found through the course of completing control experiments (for Figures 5–6) that the presence of the neurotrophin receptor had a secondary effect on the channel, it increased its expression level. Production of BDNF and expression of TrkB receptor in the nervous system has been demonstrated to be activity dependent (Balkowiec and Katz 2002). This leads to the testable hypothesis that potassium ion channel subcellular distribution or total channel expression could be altered upon upregulation of the neurotrophin signaling pathway during periods of regeneration, development, or high activity. Likewise, the downregulation of channel current could be driven both by conformational changes in the channel protein upon addition of the phosphate negative charge (Figure 6) as well as endocytosis of the channel following BDNF stimulation (Patapoutian and Reichardt 2001). Unlike the IR signaling pathway we have studied, we know that Kv1.3 and TrkB can be co-immunoprecipitated (data not shown). It is possible upon activation of TrkB by BDNF, therefore, that the channel could be downregulated both by phosphorylation and by its physical removal from the membrane as TrkB receptors are recycled. Future experiments using confocal imaging combined with surface biotinylation will be required to address such questions.

While the mechanics of the modulation that occurs at discrete biochemical sites can be satisfyingly addressed using cloned proteins in a heterologous expression system, ultimately the knowledge found in these studies must be applied to native proteins to understand neuromodulation in the brain. It is important to not solely accept the most reductionist approach of a heterologous expression system as the precise mimic of the native system. Thus, it can be very useful to use both systems as parallel evidence to support a given hypothesis. Our data demonstrate that two closely related rodent species, rat and mouse, share similar basal electrophysiological properties concerning Kv1.3-expressed currents in OB neurons. Although both from the Muridae family, it was important to establish working conditions for culturing mouse OB neurons and a description of their biophysical properties. As investigators begin to explore olfactory related gene-targeted deletions using mice and produce olfactory-derived neuronal stem cells using mice, the demand for electrophysiological characterization of cultured OB neurons will be an important launching off point. Traditionally the smaller diameter mouse neuron was not favorably utilized as a model for studying mitral and granule single cell electrophysiology, but baseline biophysical properties are necessary if gene-targeted deletions are to be studied functionally for the olfactory bulb. Our data demonstrate that activation of either receptor tyrosine kinase, IR or TrkB, initiates Kv1.3 current suppression in both species, which may be more robust for BDNF signaling in the mouse. These data are supportive of our findings for cloned Kv1.3 expressed in HEK 293 cells, with some notable comparisons. BDNF activation of TrkB causes greater current suppression of native Kv1.3 expressed in mouse neurons over that of cloned Kv1.3 in HEK cells. The degree and type of modulation by kinases has been shown to be altered in the presence of adaptor proteins, several of which are well characterized for the neurotrophin signaling pathway. Interaction with adaptor proteins expressed in native OB neurons (Cook and Fadool 2002) may accentuate how Kv1.3 is modulated by TrkB. Future studies will build on these reported protein-protein interactions to explore the degree and mechanism of neuromodulation in the OB using Kv1.3-null, IR kinase-null, and TrkB kinase-null mice in synchrony with studies that pinpoint the biochemical sites using heterologous expression.

Materials and Methods

Solutions and Reagents

Cultured olfactory bulb neuron (OBN) patch pipette solution contained (in mM): 145 KCl, 10 HEPES, 10 EGTA, 2 MgCl₂, 0.20 NaATP, 0.5 GTP (pH 7.3). Cultured OBN bath

recording solution contained (in mM): 150 NaCl, 5 KCl, 2.6 CaCl₂, 2 MgCl₂, 10 HEPES, and 100 nM tetrodotoxin (TTX) (pH 7.3). Patch pipette and bath recording solutions were formulated to maximize Kv1.3 whole-cell currents. Human embryonic kidney cell (HEK 293) patch pipette solution contained (in mM): 30 KCl, 120 NaCl, 10 HEPES, and 2 CaCl₂ (pH 7.4). HEK 293 cell recording bath solution contained (in mM): 150 KCl, 10 HEPES, 1 EGTA, and 0.5 MgCl₂ (pH 7.4). Cell lysis buffer with protease and phosphatase inhibitors (PPI solution) contained (in mM): 25 Tris (hydroxymethyl) aminomethane (pH 7.5), 250 NaCl, 5 EDTA, 1% Triton X-100, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), 10 :g/ml aprotinin, 1 :g/ml leupeptin, and 1 μ g/ml pepstatin A. Wash buffer contained (in mM): 25 Tris (pH 7.5), 250 NaCl, 5 EDTA, and 0.1% Triton X-100. TTX, a sodium channel blocker, was purchased from Calbiochem (La Jolla, CA). Margatoxin (MgTx), a Kv1.3 specific inhibitor, was a generous gift from Dr. Reid Leonard, Merck Research Laboratories. Human recombinant brain derived neurotrophic factor (BDNF) was purchased from Promega (Madison, WI). Human recombinant insulin was purchased from Roche (Indianapolis, IN). Tissue culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). All salts were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Houston, TX).

cDNA Constructs and Antibodies

Kv1.3 channel was subcloned into the multiple cloning region of pcDNA₃ (Invitrogen) using the unique restriction site *HindIII*. IR cDNA was a gift from Dr. R. Roth (Stanford University) in the pECE vector and was subcloned into the multiple cloning region of pcDNA₃ (Invitrogen) using the unique restriction sites *SalI* and *XbaI*. TrkB cDNA was a generous gift from Dr. P. Barker (McGill University) in a CMX vector. All channel and kinase coding regions were downstream from a cytomegalovirus (CMV) promoter.

AU13, a rabbit polyclonal antiserum, was generated against a 46 amino acid sequence 478 MVIEEGGMNHSAFPQTPFKT GNSTATCTTNNPNDCVNIKKIFTDV 523 representing the unique coding region of Kv1.3 between the amino terminus and transmembrane domain 1. The purified peptide was produced by Genmed Synthesis (San Francisco, CA) and the antisera was produced by Cocalico Biologicals (Reamstown, PA). This antibody was used for immunoprecipitation (1:1000) and Western blot detection (1:1500) of Kv1.3. Tyrosine phosphorylated proteins were visualized by the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology; Lake Placid, NY) and used at 1:1000 for Western blots.

Primary Cell Culture

Olfactory bulb neuron (OBN) primary cultures were prepared using a procedure modified from Huetthner and Baughman (1986) as described previously (Fadool et al. 2000) for rat with minor adjustments for mouse. Unlike previous culture conditions known to support olfactory bulb (OB) neurons from rat (Fadool et al. 2000; Tucker and Fadool 2002), neurons harvested from mice did not survive well past two or three days in vitro (DIV) in the absence of an astrocyte feeder. The total glial concentration contained in the mouse OB was apparently too low to support a co-culture of neuronal and glial cell types as is typically performed for rat. After establishing a confluent astrocyte feeder layer as an OB neuronal substrate, neurons from mice had the same longevity as that reported for rat (over 3 months) and the electrical stability of the patch recording increased, with most cells demonstrating stability over one hour.

Briefly, olfactory bulbs were harvested from four Sprague Dawley rats (Simmonson, Kilroy, CA) or six B6129SF2/J mice (Jackson Laboratories, Bar Harbor, ME) on postnatal day 1 (P1) and placed into serum-free Dulbecco's modified Eagle medium (dMEM) at 5% CO₂, 37°C. The olfactory bulbs (OB) were incubated for 25–30 minutes for mice and 50–60

minutes for rats with cysteine-activated papain (200 U, Worthington Biochemicals, Lakewood, NJ) at 37°C, 5% CO₂. To stop the enzymatic dissociation, OB were moved into dMEM containing 2% penicillin/streptomycin sulfate, 5% fetal bovine serum (FBS), and 5 mg/ml trypsin inhibitor (Roche) at 37°C, 5% CO₂ for 10 minutes. Cells were mechanically dissociated by trituration using a graded-size series of fire-polished siliconized Pasteur pipettes. The resulting neuron and glia suspension was plated onto poly-D-lysine hydrobromide (MW 49,300–53,000; Sigma) coated glass coverslips and grown in dMEM supplemented with 2% penicillin/streptomycin sulfate and 5% FBS. For mice OBN cultures, it was necessary to increase the density of the glial population by plating the neuron and glial suspension on an astrocyte feeder layer, previously established on the poly-D-lysine coated coverslips (Trombley and Blakemore 1999). Ten micromolar cytosine arabinoside (Sigma) was added to the media for 36 hours between day in vitro (*DIV*) 3–5 to stop overgrowth of dividing cells and promote better neuronal survivability.

HEK 293 Cell Culture and Transient Transfection

HEK 293 cells were maintained in minimum essential medium (MEM), 2% penicillin/streptomycin, and 10% FBS (Gibco BRL). Before transfection, cells were grown to 95% confluency, dissociated with trypsin-EDTA (Sigma) and mechanical trituration, diluted in MEM to a concentration of 600 cells/ml, and plated on Corning dishes (Catalog # 25000, Fisher Scientific). cDNA was introduced into HEK 293 cells with a lipofectamine reagent (Gibco BRL) 3–5 days after passage as previously described (Fadool et al. 1997). Briefly, cells were transfected for 4–5 hours using 4.0 µg of each cDNA construct per 60 mm dish for biochemistry or 4–4.5 hours using 1.0 µg of each cDNA construct per 35 mm dish for physiology. Plas-mid DNA with no coding insert (control vector) served as the control to equalize total µg of cDNA added to each dish. Cells were then harvested for biochemistry or used for patch-clamp recording approximately 30–48 hours post transfection.

The cDNA coding for Kv1.3 channel was from rat. Rat, human, and mouse Kv1.3 gene sequence demonstrates greater than 99% identity.

Electrophysiology of OBN Cultures

OBN cultures were voltage-clamped in the whole-cell recording configuration. Electrodes were fabricated from Jencons glass (Cat #M15/10, Jencons Limited, Bedfordshire England), fire-polished to approximately 1 µm, and coated near the tip with beeswax to reduce the electrode capacitance. Pipette resistances were between 9 and 14 MΩ. All voltage signals were generated and data were acquired using pClamp 8 software in conjunction with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The amplifier output was filtered at 5 kHz, digitized at 2–5 kHz, and stored for later analysis. Cells were held at –80 mV (V_m) and stepped to +40 mV (V_c) for a pulse duration of 400 msec, at a stimulating interval of 30 sec for peak outward current amplitude, inactivation, and deactivation measurements. Current-voltage relationships were assessed by holding the neuron at –90 mV and stepping the voltage from –80 to +40 mV in 10 mV increments for 400 msec using a 30 to 60 second interpulse interval.

Acute stimulation of OBNs was achieved by bath application of 50 ng/ml BDNF or 10 µg/ml insulin after control measurements were acquired for approximately 10 minutes. Peak outward current amplitude, inactivation, deactivation and current-voltage relationships were measured pre-stimulation (time 0), 5, 15, and 30 minutes post stimulation. Differences in these biophysical measurements were analyzed between control (time 0) and post growth factor stimulation (time 15 min) groups within single cells by paired *t*-test with statistical significance at the 95% confidence level.

In experiments using margatoxin (MgTx) to block Kv1.3 specific current, voltage-clamped OBN in the whole-cell configuration were held at -90 mV and stepped to 40 mV for 1 second every 60 seconds. Currents were recorded for $3-5$ minutes and then 100 pM of MgTx was bath applied. The current was recorded until the peak currents stabilized to the fully blocked level. Then the same neuron was treated by acute bath application of either 50 ng/ml of BDNF or 10 ug/ml insulin and recordings continued under the same voltage paradigm. Peak current amplitude was measured pre-MgTx stimulation, during block of Kv1.3 current by MgTx, and post growth factor stimulation.

All electrophysiological data were analyzed using pClamp 8.0 software in combination with the analysis packages Origin (MicroCal Software, Northampton, MA) and Quattro Pro (Borland International, Scotts Valley, CA). Data traces were subtracted linearly for leakage conductance. The inactivation of the macroscopic current was fit to the sum of two

exponentials ($y=y_0+A_1^{(-x/\tau_1)}+A_2^{(-x/\tau_2)}$) by minimizing the sums of squares, where y_0 was the Y offset, τ_1 and τ_2 were the inactivation time constants, and A_1 and A_2 were the amplitudes. The two inactivation time constants (τ) were combined by multiplying each by its weight (A) and summing ($\tau_{inact}=[(A_1^*\tau_1)+(A_2^*\tau_2)]/(A_1+A_2)$). The deactivation of the macroscopic current was fit similarly, but to a single exponential ($y=y_0+A_1^{(-x/\tau_1)}$).

Electrophysiology of HEK 293 Cells

Macroscopic currents in cell-attached membrane patches were recorded $30-48$ hr after transfection. The Kv1.3 channel expression was so robust that it was not possible to record whole-cell currents without saturating the amplifier. The diameter of the patch pipette, and hence number of ion channels sampled, was held uniform by checking the bubble number of the pipette immediately after electrode fabrication and polishing (Mittman et al. 1987). Stimulation and analysis with insulin and BDNF was the same as described for OBNs, with the exception that the hormone/trophic factor was applied in the patch pipette. This was accomplished by tip filling (0.01 mm) the patch pipette with control solution and then backfilling the pipette (~ 35 mm) with hormone/trophic factor (Fadool and Levitan 1998). The dilution of the hormone/trophic factor using this procedure would be insignificant due to the ratio of the volume (back fill/tip fill) difference that is estimated to be over 1 million.

Immunoprecipitation

For immunoprecipitation of Kv1.3 protein from transfected HEK 293 cells, cells were lysed in ice-cold PPI buffer. Lysis was continued on a Roto-Torque slow speed rotary (model 7637; Cole Palmer, Vernon Hills, IL) for 30 minutes at 4°C . The lysates were clarified by centrifugation at $14,000$ g for 10 minutes at 4°C and precleared for 1 hour with 3 mg/ml protein A-sepharose (Amersham-Pharmacia, Newark, NJ), which was followed by another centrifugation step to remove the protein A-sepharose. Kv1.3 proteins were immunoprecipitated from the clarified lysates by overnight incubation at 4°C with α -AU13 ($1:1000$), followed by a 2 hour incubation with protein A-sepharose and centrifugation as above. The immunoprecipitates were washed four times with ice-cold wash buffer. Lysates and washed immunoprecipitates were diluted in sodium dodecyl sulfate (SDS) gel loading buffer (Sambrook and Russell 2001) containing 1 mM Na_3VO_4 and stored at -20°C for subsequent analysis.

Whole-cell lysates or immunoprecipitated proteins were separated on 10% acrylamide gels by SDS-PAGE and electro-transferred to nitrocellulose blots. Blots were blocked with 5% nonfat milk (Biorad) and incubated overnight at 4°C in the presence of the antibody against Kv1.3 (α -AU13) or antiphosphotyrosine antibody (α -4G10). The blots were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody

(1:4000) (Amersham-Pharmacia) or HRP-conjugated goat anti-mouse secondary antibody (1:4000) (Sigma) for 90 minutes at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Fugi Rx film (Fisher) was used to visualize labeled proteins. The film autoradiographs were analyzed by densitometry using a Hewlett-Packard Photosmart Scanner (model 106-816, Hewlett Packard, San Diego, CA) in conjunction with Quantiscan software (Biosoft, Cambridge, England).

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FIG. 1.

Whole-cell current properties of cultured olfactory bulb (OB) neurons in rat and mouse. (A) OB neurons (Day 3 in vitro; DIV3) were patch-clamped in the whole-cell configuration. Shown is a representative family of current responses for each species, where the neuron was held (V_h) at -90 mV and stepped from -80 to $+40$ mV (V_c) in 10 mV increments for a duration of 400 msec using 30 second interpulse intervals between voltage stimulations. (B) Current-voltage relation for a population of neurons that were stimulated as in Part A but using 20 mV increases in the depolarizing voltage steps. Current was normalized to the current evoked at the $+40$ mV step. Error bars represent the standard error of the mean (s.e.m.) for 6 – 10 trials per species. ● = Rat and ■ = Mouse. DIV4. (C) Conductance measurements were calculated for the voltage protocols performed in part (B) and plotted against voltage. Maximum conductance was normalized to that of the $+40$ mV step. Same notations and sample size part (B). Solid line = best fit to a Boltzmann Equation. $V_{1/2}$ = voltage at half-activation, is calculated from the Boltzmann fit. (D) Peak current magnitude calculated from a single voltage step (V_c) from -90 to $+40$ mV was plotted for 8 – 12 OB neurons at 2 to 5 DIV. Same notations as in part (B). Solid line = best fit to a linear regression.

FIG. 2.

Brain-derived neurotrophic factor (BDNF) causes suppression of outward voltage-activated currents in mouse OB neurons. Same voltage protocols as described for Figure 1. (A) Representative family of current responses for a neuron prior (control) and following 15 minute bath application of 50 ng/ml BDNF (BDNF). DIV 3. (B) Current-voltage relation for a population of DIV3 neurons. Error bars represent the s.e.m. for 6 neurons, prior (○) and following (●) bath application of the neurotrophin. Normalization as in Figure 1.

FIG. 3. Insulin causes suppression of outward voltage-activated currents in mouse OB neurons. (A, B) Same as in Figure 2, except for neurons were treated with bath application of 10 $\mu\text{g/ml}$ insulin rather than BDNF. DIV 5.

FIG. 4.

Margatoxin (MgTx) inhibits neuromodulation of OB neurons by the ligands for receptor tyrosine kinases, IR and TrkB. Plot of the peak current magnitude of representative neurons that were held (V_h) at -90 mV and then treated with the voltage protocol in Figure 1D using a 60 second interpulse interval. One hundred picomolar of MgTx, a Kv1.3-specific ion channel blocker, was applied at the arrow and current was monitored over time () until the blocked current no longer decayed (approximately 10–15 minutes). Current was normalized to the initial current recording at time 0. Then either 50 ng/ml BDNF () or 10 μ g/ml insulin () was added at the arrow and current was monitored for 20–30 minutes or the duration of the patch recording. Here current was normalized to the final MgTx-treated current level prior to treatment with tyrosine kinase activator. Records are representative for 5 such experiments for BDNF and 6 such experiments for insulin. DIV 5.

FIG. 5.

Tyrosine phosphorylation of the Kv1.3 channel protein following BDNF stimulation. (A) Human embryonic kidney (HEK) 293 cells were transiently transfected with the cDNA for wildtype Kv1.3 plus TrkB receptor (WT Kv1.3) or cDNA for a Y to F point mutation in the channel plus TrkB receptor as indicated. Cells were acutely stimulated with control vehicle (-) or BDNF (+) supplemented media. Kv1.3 channel protein was immunoprecipitated (IP) from whole cell lysates and separated by SDS-PAGE. Proteins were electrotransferred to nitrocellulose and blotted (Blot) with the antiphosphotyrosine antibody, α -4G10 and visualized by species-specific hrp-conjugated secondary antibody. Arrows = Kv1.3 channel protein (55–68 kDa); band of immunoglobulin (IgG). (B) Total phosphorylated channel protein in part (A) was subjected to quantitative densitometry. Calculated pixel immunodensity under the control condition was normalized arbitrarily to 1.0. Mean immunodensity values are expressed as a ratio of BDNF-stimulated divided by control conditions. Each ratio value was calculated only within the same film autoradiograph to eliminate variability in epichemiluminense (ECL) exposure. Ratio value of 1.0 (dashed line) indicates no increase in phosphorylation. Number of transfections as indicated; mean $\pm$ s.e.m., * = significantly different Students *t*-test, α # 0.05, arc-sin transformation for percentage data. (C) Same techniques as in (A). Whole cell lysates were probed (Blot) with an antisera against the Kv1.3 channel protein under BDNF unstimulated conditions. (D) Histogram plot of the mean immunodensity values for four experiments as in part (C). Mean $\pm$ s.e.m., * = significantly different Students *t*-test, α # 0.05, arc-sin transformation for percentage data.

FIG. 6. BDNF-induced activation of TrkB receptor causes current suppression of Kv1.3 by targeting multiple tyrosine residues. (A) Cartoon depiction of the *Shaker* potassium channel, Kv1.3, indicating four favorable recognition motifs for tyrosine phosphorylation. A conservative Y to F single point mutation was made in each of the N and C terminal sites to eliminate the tyrosine site in the channel substrate. (B) Representative current recording of a cell-attached patch from a WT Kv1.3 + TrkB transfected HEK 293 cell that was held (V_h) at -80 mV and stepped to a single depolarizing step of $+40$ mV (V_c) for 1000 ms. Patch pipettes were tip filled with normal patch solution and then back filled with 50 ng/ml BDNF in order to present the ligand to the external face of the membrane. The control trace (Control) is the

first recording following stabilization of the patch (1–3 minutes) and the neurotrophin-treated trace (BDNF) is the same patch recorded 20 minutes later (BDNF). (C–F) Same as in (B) for various Y to F channel mutants.

FIG. 7.

Plot of time versus peak current magnitude for the experiment in Figure 6. Control trace represents a WT Kv1.3 transfected HEK 293 cell where the pipette was both tip and back filled with control patch solution (unstimulated with BDNF). Normalization is expressed as the initial current divided by current at the nth time point (I_i/I_n).

TABLE 1
Comparison of biophysical measurements in whole-cell recordings of cultured rat and mouse OB neurons (DIV 4–5)

Species	Peak current (pA)	$\tau_{\text{inactivation}}$ (ms)	$V_{1/2}$ (mV)	κ	Insulin 0 % decrease	BDNF 0 % decrease
Rat	*2986 ± 428 (8)	221 ± 30 (8)	−22 ± 3 (6)	14.2 ± 1.4 (5)	25% (12)	22% (10)
Mouse	1567 ± 250 (12)	233 ± 54 (12)	−26 ± 7 (10)	18.6 ± 2.1 (10)	29% (12)	*38% (6)

The peak current was calculated as the maximum current evoked by a 400 ms depolarizing step from −90 to +40 mV. The inactivation kinetics ($\tau_{\text{inactivation}}$) of the whole-cell current was calculated by an exponential fit to the inactivating portion of the current. $V_{1/2}$ the voltage at which half the channels were activated, was calculated by fitting normalized peak tail currents at different holding potentials to a Boltzmann Function (as in Figure 3). The slope of this function or value for steepness of voltage dependence is reported as κ .

* = Significantly different, Student's t -test, α # 0.05. Arc-Sin transformation for percentile data was used. Sample size is denoted by parenthesis; values are mean ± s.e.m.

TABLE 2
Properties and modulation by BDNF of Kv1.3 current in Kv1.3 plus TrkB co-transfected HEK293 cells

Channel construct and treatment	Peak current (pA)	$\tau_{\text{inactivation}}$ (ms)	$\tau_{\text{deactivation}}$ (ms)
WT Kv1.3	557 ± 89	521 ± 81	29 ± 7
+BDNF (n = 11)	*477 ± 76	514 ± 85	27 ± 4
YYY111-113FFF	408 ± 77	899 ± 328	48 ± 14
+BDNF (n = 8)	400 ± 88	788 ± 200	34 ± 9
Y137F Kv1.3	394 ± 80	564 ± 43	33 ± 11
+BDNF (n = 6)	405 ± 81	811 ± 375	25 ± 4
Y449F Kv1.3	541 ± 134	691 ± 133	22 ± 3
+BDNF (n = 8)	551 ± 102	606 ± 248	18 ± 2
Y479F Kv1.3	1058 ± 159	561 ± 70	22 ± 2
+BDNF (n = 7)	1080 ± 185	598 ± 77	25 ± 13

Currents were evoked by 1-s depolarizing pulses from −90 to +40 mV in the cell-attached (c.a.) configuration. Each patch pipette was tip filled with control solution and backfilled with 50 ng/ml BDNF (see Methods and Figure 7). Within patch values (mean ± s.e.m.) were taken at time 0 and 20 minute following the achievement of the c.a. configuration (+BDNF). Tau (τ) values were estimated from exponential fits to the inactivating or deactivating portions of the current.

* = BDNF-treated is significantly different from control, paired *t*-test, α # 0.05.