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FGF14 N-Terminal Splice Variants Differentially Modulate Nav1.2 and Nav1.6-Encoded Sodium Channels

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Abstract

The Intracellular Fibroblast Growth Factor (iFGF) subfamily includes four members (FGFs 11–14) of the structurally related FGF superfamily. Previous studies showed that the iFGFs interact directly with the pore-forming (α) subunits of voltage-gated sodium (Nav) channels and regulate the functional properties of sodium channel currents. Sequence heterogeneity among the iFGFs is thought to confer specificity to this regulation. Here, we demonstrate that the two N-terminal alternatively spliced FGF14 variants, FGF14-1a and FGF14-1b, differentially regulate currents produced by Nav1.2- and Nav1.6 channels. FGF14-1b, but not FGF14-1a, attenuates both Nav1.2 and Nav1.6 current densities. In contrast, co-expression of an FGF14 mutant, lacking the N-terminus, increased Nav1.6 current densities. In neurons, both FGF14-1a and FGF14-1b localized at the axonal initial segment, and deletion of the N-terminus abolished this localization. Thus, the FGF14 N-terminus is required for targeting and functional regulation of Nav channels, suggesting an important function for FGF14 alternative splicing in regulating neuronal excitability.

Introduction

Four members (FGFs 11–14) of the intracellular Fibroblast Growth Factor (iFGF) subfamily, also referred to as fibroblast growth factor homologous factors (FHF), share the core structure of FGFs, but form a distinct subfamily because they are not secreted from cells and do not interact with known FGF receptors (Itoh and Ornitz, 2008; Ornitz and Itoh, 2001). The iFGFs are highly expressed in embryonic and adult central and peripheral nervous systems, suggesting important roles in the normal development of the nervous system (Goldfarb, 2005; Smallwood

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et al., 1996). Importantly, *Fgf14*^{-/-} mice show ataxia, dystonia, and cognitive impairment (Goldfarb et al., 2007; Wang et al., 2002; Wozniak et al., 2007). In humans, an autosomal dominant missense mutation, FGF14^{F145S}, results in a progressive spinocerebellar ataxia, SCA27, and cognitive impairment (Brusse et al., 2006; Van Swieten et al., 2003).

The structure of the iFGFs has been resolved into a conserved FGF core domain and less conserved amino (N-) and carboxy (C-) termini (Smallwood et al., 1996). Diversity within the iFGF subfamily is further enhanced by N-terminal alternative splicing (Goldfarb, 2005; Munoz-Sanjuan et al., 2000; Smallwood et al., 1996; Wang et al., 2000). For example, FGF14-1a (FHF4) has a 64 amino acid N-terminus that is highly homologous to those of FGF12-1a and FGF13-1a (46% amino acid identity), whereas FGF14-1b, the most abundant form expressed in the central nervous system, has a unique 69 amino acid N-terminus (Munoz-Sanjuan et al., 2000; Wang et al., 2000). Thus, it is predicted that alternative N-termini may regulate the function of these factors.

Considerable evidence has shown that iFGFs interact directly with the pore-forming α subunit of voltage-gated sodium channels and modulate gating properties and the density of these channels (Laezza et al., 2007; Liu et al., 2001; Liu et al., 2003; Lou et al., 2005; Rush et al., 2006b; Wittmack et al., 2004). Interestingly, the functional regulation of iFGF and Nav channel pairs can be isoform-specific. For example, co-expression with FGF14-1b markedly reduces Nav1.5 and Nav1.1 current densities and regulates voltage-dependence of inactivation of the two channels in opposite directions, whereas FGF14-1a suppresses Nav1.5, but not Nav1.1, currents and depolarizes activation of Nav1.1 but not Nav1.5 (Lou et al., 2005). Moreover, while both FGF13-1a (FHF2A) and FGF13-1b (FHF2B) increase Nav1.6 current density, FGF13-1a enhances the accumulation of inactivated channels, while FGF13-1b inhibits accumulation of inactivated channels (Rush et al., 2006b); similar effects on Nav1.2 have also been observed (Lampert, Dib-Hajj and Waxman, unpublished observations).

Sodium channels Nav1.2 and Nav1.6, are abundantly expressed in the cerebellum and the hippocampus (Schaller and Caldwell, 2000, 2003; Schaller et al., 1995), two of the brain regions most affected by loss of *Fgf14* in mice or by the FGF14^{F145S} mutation in SCA27 patients (Brusse et al., 2006; Dalski et al., 2005; Goldfarb et al., 2007; Laezza et al., 2007; Van Swieten et al., 2003), suggesting that the activity of one or both of these two sodium channels might be regulated by FGF14 *in vivo*. Additionally, FGF14-1a and -1b and FGF13 have been shown to localize at the axonal initial segment (AIS) and nodes of Ranvier (Goldfarb et al., 2007; Laezza et al., 2007; Rush et al., 2006b; Wittmack et al., 2004), two distinct sub-cellular domains that contain a high density of Nav1.2 and Nav1.6 channels and that control the generation and propagation of action potentials in neurons (Peles and Salzer, 2000; Poliak and Peles, 2003). Functional analysis suggests that the FGF14^{F145S} mutant protein acts as a dominant negative and interferes with wild type FGF14 interactions with Nav channels. The expression of FGF14^{F145S} in cultured hippocampal neurons impairs neuronal excitability, a phenotype that has been linked to reduced sodium current density (Laezza et al., 2007). The lack of FGF14 in cerebellar granule and Purkinje neurons from *Fgf14*^{-/-} mice results in decreased neuronal excitability (Goldfarb et al., 2007; Shakkottai et al., 2009). These studies have revealed important roles for FGF14 in regulating neuronal activity via regulation of Nav1.2 and Nav1.6 channels.

Here, we show that both FGF14-1a and FGF14-1b interact with the intracellular C-terminal domains of Nav1.2 and Nav1.6, FGF14-1b suppresses Nav1.2 and Nav1.6 current densities, whereas FGF14-1a has no effect on Nav1.2 or Nav1.6 currents. Additionally, expression of an FGF14 variant with an N-terminus derived from that of FGF12-1b no longer is targeted to the AIS. We conclude that the FGF14 N-terminal domain contributes to both the ability of FGF14 to functionally regulate Nav channels and to the subcellular localization of FGF14 in neurons.

Methods

Plasmids

Generation and characterization of the *Fgf14-1b-Gfp*, *Fgf14-1a-Gfp* and *Fgf14-1b^{F145S}-Gfp* constructs were described previously (Laezza et al., 2007; Lou et al., 2005). *Fgf14-ΔNT-Gfp*, lacking the N-terminal domain, was amplified by PCR from *Fgf14-1b* and cloned into 5' BamHI and 3' Not I sites of the pQBI vector (Quantum Technologies). To provide FGF14-ΔNT with a transcription initiation codon, the amino acid sequence "MESK", corresponding to the N-terminal sequence encoded by the first exon of *Fgf12-1b* was added using PCR with the following primers: forward:

TACCGGTACCATGGAGAGCAAGGATCCCCAGCTCAAG; reverse:

GTTTAGCGGCCGCCTGTGGTCTTGCACTTG. Thus, the FGF14-ΔNT protein is a chimera of *Fgf12-1b* N-terminus and the core and C-terminus of *Fgf14*. The *Fgf14* plasmids with C-terminal Myc tags were described previously (Laezza et al., 2007; Lou et al., 2005). The human Nav1.2 (Scn2a, aa 1777–2005) and mouse Nav1.6 (Scn8a, aa 1765–1978) were amplified by PCR from cDNA clones and ligated in frame into the pCruz-HA plasmid (Santa Cruz, Inc.) (see Fig. 5A).

The plasmid *pcDNA3-Nav1.6R*, which encodes full-length mouse Nav1.6, was described previously (Herzog et al., 2003) and contains a substitution of tyrosine 371 by serine (Y371S) which renders Nav1.6 channels resistant to micromolar concentrations of tetrodotoxin when the channel is produced in mammalian cell lines (HEK293 and ND7/23), or in DRG neurons (Cummins et al., 2005; Herzog et al., 2003; Rush et al., 2006a; Wittmack et al., 2004; Wittmack et al., 2005).

Transfection of HEK293 and ND7/23 cells

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. HEK293 cells stably expressing rat Nav1.2 (HEK-Nav1.2 cells) were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 500 μg/ml G418 (Invitrogen) and incubated at 37°C with 5% CO₂, as previously described (Laezza et al., 2007). Cells were transfected at 90–100% confluence using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Transfection of the ND7/23 cells was as described previously (Rush et al., 2006a). Briefly, ND7/23 cells were plated at low density on 35 mm dishes 24 hours before transfection. Cells were transfected with 2 μg of Nav1.6R and *EGFP* or Nav1.6R and *Fgf14-GFP*, and electrophysiology was performed 24 hours following transfections. Only cells with robust green fluorescence and currents that larger than 50 pA were included in the analysis.

Electrophysiology

HEK293-Nav1.2—Transfected cells were dissociated and re-plated at low-density approximately 12 hr post-transfection. Recordings were obtained at room temperature (20–22°C) ~12–18 hours post-transfection using an Axopatch 1D amplifier (Axon Instruments, Union City, CA). Borosilicate glass pipettes with resistance of 1–2 MΩ or 2.5–5 MΩ were made using a P-87 Micropipette Puller (Sutter Instruments, Novato, CA). Recording solutions: extracellular (mM), 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 20 HEPES, pH 7.3; intracellular, 140 CsF, 1 EGTA, 10 NaCl, 10 HEPES, pH 7.3. After seal formation and cell break-in, membrane capacitance in each cell was calculated by integrating the area under the capacitive transients recorded in response to a 5 ms 10 mV hyperpolarizing test pulse from a holding potential of –70 mV. Capacitive transients and series resistances were compensated electronically by 80–90%. Data were acquired at 50 kHz and filtered at 5 kHz prior to digitization and storage. All

experimental parameters were controlled with Clampex (version 9.2) (Axon Instruments) using a Digidata 1322A analog-digital interface (Axon Instruments).

Voltage-dependent inward currents were evoked by brief (50–100 ms) voltage steps to test potentials between –100 mV and +60 mV from various holding potentials (between –130 mV and –90 mV). Steady-state inactivation of Nav channels was determined using a paired-pulse protocol. From the holding potential, cells were stepped to varying test potentials between –130 mV and –10 mV (prepulse of 750 ms) prior to a test pulse to –10 mV or –20 mV. Current densities were determined by dividing the amplitudes of the sodium currents (I_{Na}) at each test potential by the membrane capacitance (in the same cell). Current-voltage relations were generated by plotting peak current density as a function of the test potential. The sodium channel conductance G_{Na} was calculated using the following equation:

$$G_{Na} = I_{Na} / (V_m - E_{rev})$$

where I_{Na} is the amplitude of the current at voltage V_m , and E_{rev} is the Na^+ reversal potential. Steady-state activation curves were derived by plotting normalized G_{Na} as a function of test potential and fitted using the Boltzmann equation:

$$G_{Na} / G_{Na,Max} = 1 + e^{[(V_a - V_m)/k]}$$

where $G_{Na,Max}$ is the maximum conductance, V_a is the membrane potential at half-maximal activation, V_m is the membrane voltage and k is the slope factor.

For steady-state inactivation, normalized current amplitude ($I_{Na} / I_{Na,Max}$) at each test potential was plotted as a function of prepulse potential (V_m) and fitted using the following (Boltzmann) equation:

$$I_{Na} / I_{Na,Max} = 1 / \{ 1 + e^{[(V_h - V_m)/k]} \}$$

where V_h is the membrane potential at half-maximal inactivation and k is the slope factor.

Data analyses were performed using Clampfit 9.2 (Axon Instruments), Prism 4 software (GraphPad, San Diego, CA) and SigmaStat (Jendel Corporation). Results are presented as means $\pm$ SEM and the statistical significance of differences between groups was assessed using the Student's t test and was set at $P < 0.05$.

ND7/23 cells—Whole-cell voltage-clamp recordings (Hamill et al., 1981) from ND7/23 cells transiently transfected with Nav1.6R together with EGFP, or with one of the GFP-tagged *Fgf14* (FGF14-1a, FGF14-1b, or FGF14- Δ NT) plasmids, were performed with an EPC-9 amplifier (HEKA electronics, Lambrecht/Pfalz, Germany) using fire polished 0.5–1.5 M Ω electrodes (World Precision Instruments, Inc, Sarasota, FL, USA). The pipette solution contained (in mM): 140 CsF, 10 NaCl, 1 EGTA, and 10 HEPES; 302 mosmol (pH 7.4, adjusted with CsOH) and the extracellular bath contained (in mM): 140 NaCl, 3 KCl, 10 glucose, 10 HEPES, 1 MgCl₂, 1 CaCl₂, 0.0003 TTX; 310 mosmol (pH 7.4, adjusted with NaOH). Tetrodotoxin (TTX) was added to the bath solution to block endogenous TTX-S sodium currents, which are present in ND7/23 cells (Rush et al., 2006b; Wittmack et al., 2004). All recordings were conducted at room temperature ($\sim 21^\circ\text{C}$). The pipette potential was adjusted to zero before seal formation, and the voltages were not corrected for liquid junction potential. Capacitive transients were cancelled, and series resistances were compensated at 10 μs by 65–

95%. Leak currents were subtracted digitally online using hyperpolarizing voltage steps applied after the test pulse (P/4 procedure). Currents were acquired using Pulse software (HEKA electronics, Lambrecht/Pfalz, Germany), filtered at 10 kHz and sampled at 20–62.5 kHz; the longer inactivation protocol was sampled at 4 kHz.

Voltage-clamp recordings were obtained 4 min after establishing the whole-cell configuration. Standard current-voltage (I–V) relations were obtained using 40 ms pulses from a holding potential of –120 mV to a range of test potentials (–100 to +60 mV) in 5 mV steps with 5 seconds between pulses. Activation curves were obtained by calculating the conductance, G , at each voltage, V , as described above. To examine the voltage-dependences of steady-state inactivation of the currents, a series of 500 ms pre-pulses (–120 to –10 mV) from a holding potential of –120 mV, each followed by a 40 msec depolarization to –10 mV, were presented; voltage steps were presented at 10 sec intervals. Normalized curves were fitted using the Boltzmann equation (above).

For recovery from inactivation, two pulse protocols of 40 and 10 ms voltage steps to –10 mV and 0 mV from the holding potential of –120 mV were implemented, with variable recovery times (0.1 to 6533 ms) at –80 mV between the two pulses. Following normalization of data obtained in different cells, curves were fitted with a single rising exponential function for each cell

$$f(t) = 1 - e^{-\frac{t}{\tau}},$$

and the recovery time constant τ was calculated as the mean from all cells.

Cumulative (frequency-dependent) inactivation was examined by applying a 2 ms test pulse to –10 mV 20 times at frequencies between 0.5 Hz and 10 Hz from a holding potential of –80 mV. Current responses were normalized to the first recorded pulse and the currents at the 20th pulses were compared.

All data are presented as mean $\pm$ SEM. The statistical significance ($p < 0.05$) of observed differences among groups was determined by either Student's t-test or ANOVA using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Post hoc analysis for multiple comparisons was done using Dunnett's test.

Hippocampal neuron cultures

Hippocampal neuron cultures were prepared from embryonic day 18 (E18) rat embryos using previously described methods (Goslin et al., 1998), except that hippocampi were dissected and dissociated using papain. Briefly, following trituration through a Pasteur pipette, neurons were plated at low density ($1\text{--}5 \times 10^5$ cells/dish) on poly-L-lysine-coated coverslips in 60 mm culture dishes in MEM supplemented with 10% horse serum. After 2–4 hr, coverslips (containing neurons) were inverted and placed over a glial feeder layer in serum-free MEM with 0.1% ovalbumin, and 1 mM pyruvate (N2.1 media; Invitrogen) separated by ~1 mm wax dot spacers. The presence of the spacer prevented contact between the neurons on the coverslips and the glial feeder layer. To prevent the overgrowth of the glia, cultures were treated with cytosine arabinoside (5 μ M; Calbiochem, La Jolla, CA) at day 3 *in vitro* (DIV). Transfections were performed using Lipofectamine 2000 (Invitrogen) at DIV9.

Immunofluorescence

Rat hippocampal neurons (DIV 10) were fixed in fresh 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 15 minutes and permeabilized with 0.25% Triton-X-100. After blocking with 10% BSA for ~30 minutes at 37°C, neurons were incubated at

room temperature for 12–16 hr with a mouse monoclonal Pan-Nav channel antibody (diluted 1:100, Sigma, St Louis, MO) and polyclonal chicken anti- β IV spectrin (diluted 1:1000, gift from Dr. M. Komada (Tokyo Institute of Technology, Tokyo, Japan). All antibodies were diluted (to the concentrations noted) in PBS containing 3% BSA. Following incubations with the primary antibody combinations, neurons were washed three times in PBS and incubated for 2 hr at 37°C with appropriate secondary antibodies: Alexa 647-conjugated goat-anti-mouse IgG1 (1:500) together with aminomethylcoumarin (AMCA)-conjugated-goat-anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA) for the cells stained with the mouse monoclonal pan Nav channel antibody and the rabbit polyclonal anti-MAP2 antibodies. Coverslips were then washed (three times) with PBS and mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabicyclo[2,2,2] octane). Images were acquired using an Axio Imager epifluorescence microscope (Zeiss, Oberkochen, Germany) with a 63 $\times$ objective. Images were acquired with an Axio Cam MRm using the Axio Vision software (Zeiss).

Immunoprecipitation

Transfected HEK293 cells were washed with PBS and lysed in lysis buffer containing (in mM): 20 Tris-HCl, 150 NaCl, 1% NP-40. Protease inhibitor cocktail (set #3, Calbiochem) was added immediately before cell lysis. Cell extracts were collected and sonicated for 20 sec and centrifuged at 4°C, 15,000g for 15 min. Rabbit anti-Myc-agarose beads (Sigma, St. Louis, MO) were incubated with the supernatants 2 hours at 4°C with agitation and then washed 3 times in PBS, 0.1 % NP-40 buffer before adding 2x sample buffer containing 50 mM tris(2-carboxyethyl)phosphine (TCEP) and 4M urea. Mixtures were heated for 10 min at 65°C and resolved on 4–15% polyacrylamide gels (BioRad, Hercules, CA). Resolved proteins were transferred to PVDF membranes (Millipore, Bedford, MA) for 1.5 hours at 4°C and blocked in tris-buffered saline (TBS) with 3% nonfat dry milk and 0.1% Tween-20. Membranes were then incubated in blocking buffer containing the monoclonal anti-Myc (1:1000) or anti-HA (1:1000, Sigma, H9856) antibody overnight. Washed membranes were incubated with goat anti-mouse-HRP (1:5000) and visualized with the Super Signal Femto chemiluminescence substrate (Pierce, Rockford, IL).

Image analysis

For the quantitative analysis of the AIS expression of GFP, FGF14-1a-GFP, FGF14-1b-GFP, or FGF14- Δ NT-GFP, a mask of the axonal initial segment was first generated by thresholding the β IV spectrin images. The mask of the corresponding region was then applied to the unthresholded GFP images. Fluorescence intensity in dendrites was directly measured on the GFP images. The AIS enrichment index (AEI) was defined as the ratio of total fluorescence intensity/area in the AIS divided by the total fluorescence intensity/area in the dendrites (Laezza et al., 2007; Lou et al., 2005). Analysis was done using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2008).

Results

FGF14 regulates heterologously expressed Nav1.2 current

To determine whether FGF14 modulates the functional properties of Nav1.2-encoded currents, *Gfp*, *Fgf14-1a-Gfp* or *Fgf14-1b-Gfp* was transiently transfected into an HEK293 cell line stably expressing Nav1.2 (Laezza et al., 2007). In control cells expressing GFP, robust fast activating and inactivating inward voltage-dependent sodium currents, I_{Na} , were recorded in response to depolarizing voltage steps (Fig. 1A). In Nav1.2 cells co-expressing FGF14-1b-GFP, however, mean $\pm$ SEM ($n = 28$) peak I_{Na} density was significantly ($p < 0.001$) lower (-91 ± 11 pA/pF) than in GFP-expressing control cells (-242 ± 20 pA/pF, $n = 37$). Interestingly, co-expression of FGF14-1a-GFP, or of a construct lacking the FGF14 N-terminus, FGF14- Δ NT-GFP, which

resembles the 1b splice forms of other iFGFs (Lou et al., 2005), did not result in any measurable differences in Nav1.2 current densities (Fig. 1A,D, Table 1).

The voltage-dependences of I_{Na} activation and steady-state inactivation were determined by plotting normalized current amplitudes as a function of the test potential (activation) or the pre-pulse potential (inactivation), as detailed in Methods. These analyses revealed that expression of FGF14-1b-GFP resulted in a small (+4 mV), but statistically significant ($p < 0.05$) depolarizing shift in the voltage dependence of Nav1.2 activation, without measurably affecting steady-state inactivation of the current, whereas expression of FGF14-1a-GFP produced a statistically significant ($p < 0.01$) depolarizing shift (+9 mV) in the voltage-dependence of inactivation. Expression of FGF14- Δ NT-GFP, in contrast, resulted in a statistically significant ($p < 0.001$) hyperpolarizing shift (−5 mV) in the voltage-dependence of steady-state inactivation without changing the properties of activation. The N-terminal domain of FGF14, therefore, influences the modulatory effects of FGF14 on Nav1.2-encoded currents.

FGF14 regulates heterologously expressed Nav1.6R currents

Similar experiments were completed to examine the effects of FGF14 on Nav1.6R encoded currents. The effects of the FGF14 N-terminal variants on the properties of Nav1.6R current were examined in the neuronal cell line, ND7/23, transiently transfected with Nav1.6R, a tetrodotoxin-resistant variant of Nav1.6 (Herzog et al., 2003), together with GFP or one of the GFP-tagged FGF14 variants indicated above. Recordings were obtained from GFP-positive cells in the presence of 300 nM TTX to block the endogenous TTX-S currents present in ND7/23 cells (Wittmack et al., 2004). Rapidly activating and inactivating sodium channel currents were recorded in all cells (Fig. 2A). In ND7/23 cells co-expressing Nav1.6R and GFP, Nav1.6R currents (-74 ± 8 pA/pF; $n=30$) with properties similar to those recorded from DRG neurons transfected with Nav1.6R (Herzog et al., 2003), were obtained. Similar peak Nav1.6R current density (-81 ± 24 pA/pF; $n=12$) was obtained in cells co-expressing GFP or FGF14-1a-GFP and Nav1.6R (Fig. 2A,D). In contrast, co-expression of FGF14-1b with Nav1.6R resulted in significantly ($p < 0.01$) reduced current densities (-14 ± 3 pA/pF; $n=15$). Indeed, the residual Nav1.6R current in cells co-expressing FGF14-1b-GFP should be considered an overestimate, as Nav1.6R currents were not detectable in 40 of 55 GFP-positive ND7/23 cells co-expressing FGF14-1b-GFP and Nav1.6R. In contrast, Nav channel currents < 50 pA were only observed in 2 of 26 GFP-positive ND7/23 cells expressing Nav1.6R and FGF14-1a-GFP and in 2 of 14 GFP-positive ND7/23 cells expressing Nav1.6R and FGF14- Δ NT-GFP. Interestingly, co-expression of FGF14- Δ NT-GFP with Nav1.6R significantly ($p < 0.01$) increased current density by almost 3 fold, to 168 ± 20 pA/pF ($n=12$; Fig. 2A,D).

Experiments were also completed to investigate the effects of FGF14-1a-GFP, FGF14-1b-GFP and FGF14- Δ NT on activation and steady-state fast-inactivation of Nav1.6R currents. As shown in Fig. 2C, E and Table 2, Nav1.6R activation was not affected by co-expression of FGF14-1a-GFP or FGF14-1b-GFP, although there was a depolarizing shift in the voltage-dependence of activation when Nav1.6R and FGF14- Δ NT were co-expressed. In contrast, all FGF14 constructs induced a significant depolarizing shift in the voltage-dependence of steady-state inactivation (Fig. 2B, F).

Fgf14-1a modulates Nav1.6R recovery kinetics

Nav1.6 channels play an important role in maintaining high frequency firing in cerebellar Purkinje cells (Levin et al., 2006), and loss of *Fgf14* severely impairs the spontaneous firing properties of Purkinje neurons (Shakkottai et al., 2009). Additional experiments were completed, therefore, to explore further the potential functional effects of FGF14 on Nav1.6R-encoded Na currents. The expression of Nav1.6R in ND7/23 cells produced a current that recovers from fast-inactivation with properties similar to those of DRG neurons (Herzog et al.,

2003). Previous studies have shown that FGF13-1a-GFP slowed Nav1.6R recovery from inactivation (Rush et al., 2006b), whereas FGF13-1b-GFP did not (Wittmack et al., 2004). We investigated the effects of FGF14 on the kinetics of recovery from inactivation of Nav1.6R currents using a two pulse protocol, as shown in Fig. 3A. Cells were held at -120 mV and two depolarizing steps to -10 mV were applied. These two pulses were separated by a hyperpolarizing voltage step to -80 mV of variable duration, during which channels recovered from fast-inactivation. Cells co-expressing Nav1.6R and GFP recovered quickly, with the majority of the current being fully available after 100 ms. Co-expression of Nav1.6R with FGF14-1b-GFP or FGF14- Δ NT resulted in recovery from inactivation with similar kinetics. In contrast, co-expression of FGF14-1a-GFP with Nav1.6R resulted in a much slower recovery from inactivation, with full recovery requiring more than one second (Fig. 3A). Analyses of the recovery data (Fig. 3B) revealed that the mean time constants of Nav1.6R recovery were similar in cells co-expressing Nav1.6R with GFP (17 ± 1.1 ms; $n = 23$); FGF14-1b-GFP (50 ± 22 ms; $n = 7$) and FGF14- Δ NT-GFP (13 ± 1 ms; $n = 10$), as illustrated in Fig. 3B. The large variability in the case of FGF14-1b is related to the very small Nav1.6R currents that are recorded under this condition. In contrast, co-expression with FGF14-1a-GFP caused a significant ($p < 0.05$) increase in the time constant of Nav1.6R current recovery: 346 ± 19 ms ($n = 9$) (Fig. 3B).

The marked slowing of Nav1.6R current recovery observed in cells expressing FGF14-1a-GFP, compared to the other FGF14 proteins in these experiments (Fig. 3), suggested that the effects of the FGF14 variants on Nav1.6R currents would be particularly dramatic during repetitive stimulation. To test this hypothesis directly, the peak Nav1.6R currents in the presence of the different FGF14 proteins were measured during short test pulses to -10 mV at a range of stimulation frequencies (0.5–10 Hz) from a holding potential of -80 mV (Fig. 4). These experiments revealed that co-expression of FGF14-1a-GFP results in marked reductions in peak Nav1.6R current amplitudes at all stimulation frequencies tested. At a stimulation frequency of 5 Hz for example, the peak current amplitude was reduced markedly (by $\sim 50\%$) in the second stimulus of the train in cells co-expressing FGF14-1a-GFP, whereas current amplitudes were unchanged in cells co-expressing GFP, FGF14-1b-GFP or FGF14- Δ NT-GFP (Fig. 4A). Co-expression with FGF14-1b-GFP prevented the slight decrease in peak Nav1.6R currents observed at 5 Hz in cells expressing Nav1.6R and GFP. In the presence of FGF14-1a-GFP, the decline in Nav1.6R current amplitudes was even more dramatic at higher stimulation frequencies, reaching 80% at a frequency of 10 Hz (Fig. 4B, Table 3). Thus, the N-terminal domain of FGF14 is essential not only for modulating Nav1.6 current density, but also for regulating recovery from inactivation of this channel and, therefore, its availability during repetitive stimulation.

FGF14 interactions with C-terminal domains of Nav channels

Different members of the iFGF family have been shown to interact directly with the intracellular C-terminal tails of Nav1.5, Nav1.6 and Nav1.9 (Goetz et al., 2009; Liu et al., 2001; Wittmack et al., 2004). The C-termini of Nav1.2 and Nav1.6 are highly conserved, especially in the membrane proximal region (Fig. 5A). Previous studies on FGF12-1b-GFP demonstrated that this interaction is mediated by amino acid residues 1–41 in the N-terminal end of the conserved FGF core domain (Liu et al., 2001; Liu et al., 2003). These residues show 71% identity with the FGF14 core domain and 78% amino acid identity with the FGF13-1b core domain, suggesting that a common binding region might mediate interaction with Nav channels.

We investigated whether N-terminal FGF14 splice variants interact with the C-terminal tails of the sodium channels Nav1.2 and Nav1.6. For these experiments, *Fgf14-1a-Myc*, *Fgf14-1b-Myc*, *Fgf14- Δ NT-Myc* or *Fgf14-1b^{F145S}-Myc* were co-transfected together with a plasmid

expressing the C-terminal tail of Nav1.2 or Nav1.6 tagged with the HA epitope (HA-Nav1.2-C-tail and HA-Nav1.6-C-tail) into HEK293 cells. As shown in Figure 5B, the HA-Nav1.2 C-terminal tail immunoprecipitated with FGF14-1b-Myc, FGF14-1a-Myc, and FGF14-ΔNT-Myc but not with the negative control, FGF14^{F145S}-Myc, which has been previously shown not to interact with full length Nav1.2 (Laezza et al., 2007). Similarly, the HA-Nav1.6 C-terminal tail immunoprecipitated with FGF14-1b-Myc and FGF14-1a-Myc, but not with FGF14^{F145S}-Myc. Interestingly, although FGF14-ΔNT-Myc immunoprecipitated with the HA-Nav1.2 C-tail, this protein was not as efficient in immunoprecipitating the HA-Nav1.6-C-tail. This was unexpected because FGF14-ΔNT-Myc co-expression significantly (and by nearly threefold) increased Nav1.6R current densities (Fig. 2A–D). One possible explanation for this apparent discrepancy is that the interaction of FGF14-ΔNT-Myc with the C-terminal tail of Nav1.6R is of lower affinity (than for the other FGF14 constructs) and was, therefore, not detected in the immunoprecipitation assay. Alternatively, FGF14-ΔNT-Myc could interact with another region (i.e., other than the C terminal tail) of the Nav1.6 protein. Collectively, these experiments demonstrate that FGF14-1a-Myc and FGF14-1b-Myc interact with the C-terminal tails of both Nav1.2 and Nav1.6 and suggest that, despite the differences in primary sequence, both FGF14 splice forms retain the ability to interact with a common intracellular C-terminus of Nav channels.

The FGF14 N terminus is required for localization to the axon initial segment

In neurons, Nav1.2 and Nav1.6 channels are concentrated at the axon initial segment (AIS) and at nodes of Ranvier (Boiko et al., 2001; Boiko et al., 2003; Kaplan et al., 2001). Interestingly, recent evidence indicates that native FGF14 is concentrated at the AIS in hippocampal neurons (Laezza et al., 2007) and co-localizes with native Nav channels and with β-IV spectrin, an AIS marker (Komada and Soriano, 2002; Nishimura et al., 2007). Similar to FGF14, endogenous FGF13 has also been shown to co-localize with Nav channels at the AIS and at nodes of Ranvier (Goldfarb et al., 2007; Wittmack et al., 2004), raising the possibility that iFGFs might share common molecular determinants important for their sub-cellular localization.

The results presented above demonstrate that the alternatively spliced N-terminal domains of FGF14 confer different regulatory effects on Nav current densities and gating properties. Intriguingly, however, previous findings indicate that both FGF14-1a-GFP and FGF14-1b-GFP are enriched at the AIS when heterologously expressed in hippocampal neurons (Lou et al., 2005), suggesting that the sub-cellular targeting and the functional effects (on Nav channels) of FGF14 might be mediated by different protein domains.

To assess whether the N-terminal domain of FGF14 regulates the sub-cellular targeting of this factor, GFP, FGF14-ΔNT-GFP, FGF14-1a-GFP or FGF14-1b-GFP was transfected into cultured hippocampal neurons. Neurons were fixed and immunolabeled with an anti β-IV spectrin antibody to identify the AIS and imaged for GFP fluorescence (Fig. 6). As previously demonstrated, these experiments revealed that FGF14-1a-GFP and FGF14-1b-GFP were enriched at the AIS (Laezza et al., 2007; Lou et al., 2005) and co-localized with endogenous β-IV spectrin (Fig. 6D–F, G–I). In contrast, FGF14-ΔNT-GFP (Fig. 6J–L) was uniformly distributed throughout the cell soma, dendrites and axon, showing no enrichment at the AIS (Fig. 6M), similar to GFP expressing neurons (Fig. 6A–C). The simplest interpretation of these findings is that the presence of the N-terminal domain is necessary for proper targeting of FGF14 to the AIS.

Discussion

FGF14-1b potentially reduces heterologously expressed Nav1.2 and Nav1.6 current densities

Accumulating evidence has established a link between the iFGFs and the regulation of Nav channel activity and localization (Laezza et al., 2007; Liu et al., 2003; Lou et al., 2005; Rush et al., 2006b; Wittmack et al., 2004). However, the functional consequence of these interactions on sodium currents are both iFGF and Nav channel isoform-dependent. *In vitro*, FGF12-1b, FGF13-1a and FGF13-1b have all been shown to either have no effect or to up-regulate Nav1.5 or Nav1.6 current amplitudes in heterologous expression systems (Liu et al., 2003; Rush et al., 2006b; Wittmack et al., 2004). In contrast, FGF14-1b, the major neuronal form of *Fgf14*, was shown previously to inhibit heterologously expressed Nav1.1 and Nav1.5 currents (Lou et al., 2005; Wang et al., 2000). These observations suggest that the physiological activity of FGF14-1b may be fundamentally different from FGF14-1a and other iFGFs and further that the unique FGF14-1b N-terminus is responsible for the profound difference in functional properties.

The results of the experiments presented here demonstrate that Nav1.2 and Nav1.6 current densities are reduced in cells co-expressing FGF14-1b, whereas FGF14-1a co-expression did not measurably affect peak Nav1.2 or Nav1.6 current densities. We also show here that both FGF14-1a and FGF14-1b interact with the intracellular C-terminal domains of Nav1.2 and Nav1.6 channels and that both forms of FGF14 are targeted to the AIS of hippocampal neurons. Taken together with the results of previous studies showing that FGF14-1b interacts with Nav1.1 and attenuates Nav1.1 currents (Lou et al., 2005), the results here demonstrate that FGF14-1b can interact with and similarly modulate the properties of all Nav channels thus far tested. The function of FGF14-1b in attenuating the amplitudes of heterologously expressed neuronal Nav channels appears to be unique compared to other iFGFs and to FGF14-1a, in the HEK 293 and ND7/23 cell expression systems. The N-terminal 1b domain of FGF14 appears essential, as substitution with the similarly sized 1a domain, or deletion of the N-terminus, abolishes the ability of FGF14 to reduce sodium current densities. These results also suggest that FGF14-1b may also play an important role in the regulation of neuronal Nav channels *in vivo*.

In addition to the distinct effects on Nav channel current densities, FGF14-1a and FGF14-1b also differentially modify the voltage-dependences of activation and inactivation of neuronal Nav channel currents. Co-expression with FGF14-1a (but not FGF14-1b), for example, shifts the voltage-dependence of inactivation of Nav1.2 currents and FGF14-1b (but not FGF14-1a) modifies the voltage-dependence of Nav1.2 current activation. Both FGF14-1a and FGF14-1b, however, produce substantial (~+10 mV) shifts in Nav1.6R current inactivation; shifts that could alter Nav1.6R channel availability near the resting potential and impact neuronal excitability directly. Interestingly, the observed effect is similar to that reported previously for FGF13-1a (Rush et al., 2006b). These data suggest that there may be some conserved functions among the 1a N-terminal iFGF isoforms.

Other studies showed that FGF14-1b, but not FGF14-1a, suppressed Nav1.1 currents expressed in heterologous cells (Lou et al., 2005; Rusconi et al., 2009). However, both FGF14-1a and FGF14-1b effectively inhibited Nav currents carried by Nav1.1-R191G, a C-terminal mutant found in General Epilepsy Febrile Syndrome (Rusconi et al., 2009). This suggests that interactions between different iFGF isoforms and the C-terminal domain of Nav channels may contribute to phenotypes associated with human sodium channelopathies. Future studies will be needed to address possible interactions between iFGFs and mutations in other Nav channel α subunits.

FGF14-1b and FGF14-1a interact directly with the C termini of Nav1.2 and Nav1.6

It has previously been demonstrated that FGF14-1a and FGF14-1b directly interact with full-length Nav1.1, Nav1.2 and Nav1.5 in heterologous expression systems and with native Nav channels expressed in the Neuro2a neuroblastoma cell line (Laezza et al., 2007; Lou et al., 2005). FGF12 and FGF13 also reportedly interact directly with Nav channels (Liu et al., 2001; Liu et al., 2003; Wittmack et al., 2004). The interaction between FGF13-1b and Nav1.6 is mediated via the intracellular C-terminal domain of Nav1.6 (Goetz et al., 2009; Wittmack et al., 2004). To begin to understand the structure/function relationship between FGF14 and neuronal Nav channels, experiments were designed to test whether different forms of FGF14 could interact with the C-terminal domains of Nav1.2 and Nav1.6. The results of these experiments revealed that FGF14-1a and FGF14-1b interact with the C-terminal domains of both Nav1.2 and Nav1.6 α subunits.

Previous data suggest that the membrane-proximal segment of the C-terminus of Nav1.5 and Nav1.9, which has ~70% sequence identity among Nav channels, is important for the interaction with FGF12 (Liu et al., 2001; Liu et al., 2003). In addition to direct effects on Nav channel activation and inactivation, the interaction with the iFGFs might be important in regulating the stability or the trafficking to the cell surface of assembled Nav channels, effects that could account for the observed changes in sodium current densities observed in cells co-expressing FGF14-1b. For example, iFGF binding could modulate the function of a potential di-leucine motif in the proximal region of the Nav1.2 C-terminal domain (Fig. 5A) that may contribute to trafficking of the channel to the axon (Garrido et al., 2001). Alternatively, iFGF binding may affect a PY motif (-PPSY) (Fig. 5A), which forms a binding site for Nedd4 and Nedd4-2, ubiquitin-protein ligases implicated in the endocytic removal of the channel from the plasma membrane (Fotia et al., 2004; Rougier et al., 2005). Recent evidence indicates that co-expression of the FGF14 mutant protein FGF14^{F145S}, identified in a family afflicted with an autosomal dominant form of spinocerebellar ataxia (SCA27) (Brusse et al., 2006; Dalski et al., 2005), decreases expression of Nav channels at the AIS through a dominant negative effect on wild type FGF14, preventing endogenous wild type FGF14 from interacting with Nav channels (Laezza et al., 2007). This suggests a potential active role for endogenous FGF14 in regulating the stability and/or the trafficking of Nav channels to specific subcellular compartments. Further experiments will be needed to determine directly whether FGF14-Nav α subunit interactions mediate trafficking of Nav channels.

AIS localization of FGF14-1b and FGF14-1a in hippocampal neurons

Endogenous FGF13 and FGF14 are localized to the AIS and co-localize with Nav channels in cerebellar granule and hippocampal neurons, respectively, suggesting that iFGFs might play a role in stabilizing or targeting native Nav channels to specific subcellular compartments (Goldfarb et al., 2007; Laezza et al., 2007). For example, iFGFs might affect the interaction of Nav channels with other proteins, such as ankyrin-G (Garrido et al., 2001; Garrido et al., 2003a; Garrido et al., 2003b). Alternatively, iFGFs, which interact with JIP2, which in turn interacts with kinesin light chain (Hirokawa and Takemura, 2005; Schoorlemmer and Goldfarb, 2002; Verhey et al., 2001), may play a role in the regulation of axonal transport. Whether the interaction of iFGFs with the C-terminal domain of Nav channels is sufficient to mediate localization of the channels remains to be determined.

An important finding of the present study is that the presence of the 1a or 1b N-terminal domain of FGF14 is necessary to control the sub-cellular targeting of FGF14 in hippocampal neurons. Indeed, deletion of the N-terminal domain eliminates the specific targeting of FGF14 to the AIS. Thus, the N-terminal domain of FGF14 not only modulates the regulation of Nav channel function by FGF14, but also has a distinct role in the sub-cellular localization of FGF14. One unexpected result is that both FGF14-1a and FGF14-1b are targeted to the AIS, even though

the 1a and 1b protein domains share few conserved sequences (Lou et al., 2005; Wang et al., 2000). This suggests that specific sequences in the N-terminal domains are not necessary for AIS localization. Future experiments, focused on testing this hypothesis directly are warranted.

Taken together with previous work, the results presented here clearly demonstrate a direct role for FGF14 in regulating both the densities and the biophysical properties of Nav1.2 and Nav1.6 currents in heterologous cells. As previously reported, however, there is a dramatic difference between the effects of FGF14 on Nav current densities in heterologous cells and in neurons. In heterologous cells, FGF14-1b reduces sodium currents, and similar results have now been obtained for all channels tested. In contrast, in cultured hippocampal neurons, overexpression of FGF14-1a or FGF14-1b results in markedly increased Nav current densities (Lou et al., 2005), whereas genetic ablation of FGF14 in *Fgf14*^{-/-} mice or expression of the SCA27-linked FGF mutation, *FGF14*^{F145S}, results in impairment of excitability in the cerebellum and in the hippocampus, as well as in isolated hippocampal neurons *in vitro* (Goldfarb et al., 2007; Laezza et al., 2007; Shakkottai et al., 2009). These results suggest that endogenous FGF14 functions to promote activity of neuronal Nav channels rather than suppress activity as observed in heterologous cells. This paradox likely results from the expression of different sets of proteins in heterologous cells and neurons that could differentially affect the end result of iFGF activity. Alternatively, the iFGFs or the sodium channels might be modified post-translationally and these modifications may be different in neurons and in heterologous cells.

Taken together with findings detailed in several recent studies, the results presented here emphasize the potential complexities of iFGF-mediated effects on Nav channels and on the regulation of neuronal excitability. In addition, cell type specific differences in iFGF expression, redundant and/or competing actions of the iFGFs, and/or heteromeric assembly among the various iFGFs might add further complexity to the mechanisms of iFGF action in neurons.

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References

- Boiko T, Rasband MN, Levinson SR, Caldwell JH, Mandel G, Trimmer JS, Matthews G. Compact myelin dictates the differential targeting of two sodium channel isoforms in the same axon. *Neuron* 2001;30:91–104. [PubMed: 11343647]
- Boiko T, Van Wart A, Caldwell JH, Levinson SR, Trimmer JS, Matthews G. Functional specialization of the axon initial segment by isoform-specific sodium channel targeting. *J. Neurosci* 2003;23:2306–2313. [PubMed: 12657689]
- Brusse E, de Koning I, Maat-Kievit A, Oostra BA, Heutink P, van Swieten JC. Spinocerebellar ataxia associated with a mutation in the fibroblast growth factor 14 gene (SCA27): A new phenotype. *Mov. Disord* 2006;21:396–401. [PubMed: 16211615]
- Cummins TR, Dib-Hajj SD, Herzog RI, Waxman SG. Nav1.6 channels generate resurgent sodium currents in spinal sensory neurons. *FEBS Lett* 2005;579:2166–2170. [PubMed: 15811336]
- Dalski A, Atici J, Kreuz FR, Hellenbroich Y, Schwinger E, Zuhlke C. Mutation analysis in the fibroblast growth factor 14 gene: frameshift mutation and polymorphisms in patients with inherited ataxias. *Eur. J. Hum. Genet* 2005;13:118–120. [PubMed: 15470364]

- Fotia AB, Ekberg J, Adams DJ, Cook DI, Poronnik P, Kumar S. Regulation of neuronal voltage-gated sodium channels by the ubiquitin-protein ligases Nedd4 and Nedd4-2. *J. Biol. Chem* 2004;279:28930–28935. [PubMed: 15123669]
- Garrido JJ, Fernandes F, Giraud P, Mouret I, Pasqualini E, Fache MP, Jullien F, Dargent B. Identification of an axonal determinant in the C-terminus of the sodium channel Na(v)1.2. *EMBO J* 2001;20:5950–5961. [PubMed: 11689435]
- Garrido JJ, Fernandes F, Moussif A, Fache MP, Giraud P, Dargent B. Dynamic compartmentalization of the voltage-gated sodium channels in axons. *Biol. Cell* 2003a;95:437–445. [PubMed: 14597261]
- Garrido JJ, Giraud P, Carlier E, Fernandes F, Moussif A, Fache MP, Debanne D, Dargent B. A targeting motif involved in sodium channel clustering at the axonal initial segment. *Science* 2003b;300:2091–2094. [PubMed: 12829783]
- Goetz R, Dover K, Laezza F, Shtraizent N, Huang X, Tchetchik D, Eliseenkova AV, Xu CF, Neubert TA, Ornitz DM, Goldfarb M, Mohammadi M. Crystal structure of a fibroblast growth factor homologous factor (FHF) defines a conserved surface on FHFS for binding and modulation of voltage-gated sodium channels. *J. Biol. Chem.* 2009 in press.
- Goldfarb M. Fibroblast growth factor homologous factors: evolution, structure, and function. *Cytokine Growth Factor Rev* 2005;16:215–220. [PubMed: 15863036]
- Goldfarb M, Schoorlemmer J, Williams A, Diwakar S, Wang Q, Huang X, Giza J, Tchetchik D, Kelley K, Vega A, Matthews G, Rossi P, Ornitz DM, D'Angelo E. Fibroblast Growth Factor Homologous Factors Control Neuronal Excitability through Modulation of Voltage-Gated Sodium Channels. *Neuron* 2007;55:449–463. [PubMed: 17678857]
- Goslin, K.; Asmussen, H.; Banker, G. Rat hippocampal neurons in low-density culture. In: Banker, G.; Goslin, K., editors. *Culturing Nerve Cells*. Cambridge: MIT Press; 1998. p. 339–370.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 1981;391:85–100. [PubMed: 6270629]
- Herzog RI, Cummins TR, Ghassemi F, Dib-Hajj SD, Waxman SG. Distinct repriming and closed-state inactivation kinetics of Nav1.6 and Nav1.7 sodium channels in mouse spinal sensory neurons. *J. Physiol. (Lond)* 2003;551:741–750. [PubMed: 12843211]
- Hirokawa N, Takemura R. Molecular motors and mechanisms of directional transport in neurons. *Nat. Rev. Neurosci* 2005;6:201–214. [PubMed: 15711600]
- Itoh N, Ornitz DM. Functional Evolutionary History of the Mouse Fgf Gene Family. *Dev. Dyn* 2008;237:18–27. [PubMed: 18058912]
- Kaplan MR, Cho MH, Ullian EM, Isom LL, Levinson SR, Barres BA. Differential control of clustering of the sodium channels Na(v)1.2 and Na(v)1.6 at developing CNS nodes of Ranvier. *Neuron* 2001;30:105–119. [PubMed: 11343648]
- Komada M, Soriano P. [Beta]IV-spectrin regulates sodium channel clustering through ankyrin-G at axon initial segments and nodes of Ranvier. *J. Cell Biol* 2002;156:337–348. [PubMed: 11807096]
- Laezza F, Gerber BR, Lou J, Kozel MA, Hartman H, Craig AM, Ornitz DM, Nerbonne JM. The Fgf14 (F145S) mutation disrupts the interaction of FGF14 with voltage-gated Na⁺ channels and impairs neuronal excitability. *J. Neurosci* 2007;27:12033–12044. [PubMed: 17978045]
- Levin SI, Khaliq ZM, Aman TK, Grieco TM, Kearney JA, Raman IM, Meisler MH. Impaired motor function in mice with cell-specific knockout of sodium channel Scn8a (NaV1.6) in cerebellar purkinje neurons and granule cells. *J. Neurophysiol* 2006;96:785–793. [PubMed: 16687615]
- Liu C, Dib-Hajj SD, Waxman SG. Fibroblast growth factor homologous factor 1B binds to the C terminus of the tetrodotoxin-resistant sodium channel rNav1.9a (NaN). *J. Biol. Chem* 2001;276:18925–18933. [PubMed: 11376006]
- Liu CJ, Dib-Hajj SD, Renganathan M, Cummins TR, Waxman SG. Modulation of the cardiac sodium channel Na(v)1.5 by fibroblast growth factor homologous factor 1B. *J. Biol. Chem* 2003;278:1029–1036. [PubMed: 12401812]
- Lou JY, Laezza F, Gerber BR, Xiao M, Yamada KA, Hartmann H, Craig AM, Nerbonne JM, Ornitz DM. Fibroblast Growth Factor 14 is an Intracellular Modulator of Voltage-Gated Sodium Channels. *J. Physiol. (Lond)* 2005;569:179–193. [PubMed: 16166153]

- Munoz-Sanjuan I, Smallwood PM, Nathans J. Isoform diversity among fibroblast growth factor homologous factors is generated by alternative promoter usage and differential splicing. *J. Biol. Chem* 2000;275:2589–2597. [PubMed: 10644718]
- Nishimura K, Akiyama H, Komada M, Kamiguchi H. betaIV-spectrin forms a diffusion barrier against L1CAM at the axon initial segment. *Mol. Cell. Neurosci* 2007;34:422–430. [PubMed: 17223356]
- Ornitz DM, Itoh N. Fibroblast growth factors. *Genome Biology* 2001;2 REVIEWS3005.
- Peles E, Salzer JL. Molecular domains of myelinated axons. *Curr. Opin. Neurobiol* 2000;10:558–565. [PubMed: 11084317]
- Poliak S, Peles E. The local differentiation of myelinated axons at nodes of Ranvier. *Nat. Rev. Neurosci* 2003;4:968–980. [PubMed: 14682359]
- Rougier JS, van Bemmelen MX, Bruce MC, Jespersen T, Gavillet B, Apotheloz F, Cordonier S, Staub O, Rotin D, Abriel H. Molecular determinants of voltage-gated sodium channel regulation by the Nedd4/Nedd4-like proteins. *Am. J. Physiol. Cell Physiol* 2005;288:C692–C701. [PubMed: 15548568]
- Rusconi R, Combi R, Cestele S, Grioni D, Franceschetti S, Dalpra L, Mantegazza M. A rescuable folding defective Na(v)1.1 (SCN1A) sodium channel mutant causes GEFS+: Common mechanism in Na(v)1.1 related epilepsies? *Hum. Mutat.* 2009 in press.
- Rush AM, Dib-Hajj SD, Liu S, Cummins TR, Black JA, Waxman SG. A single sodium channel mutation produces hyper- or hypoexcitability in different types of neurons. *Proc. Natl. Acad. Sci. U. S. A* 2006a;103:8245–8250. [PubMed: 16702558]
- Rush AM, Wittmack EK, Tyrrell L, Black JA, Dib-Hajj SD, Waxman SG. Differential modulation of sodium channel Na(v)1.6 by two members of the fibroblast growth factor homologous factor 2 subfamily. *Eur. J. Neurosci* 2006b;23:2551–2562. [PubMed: 16817858]
- Schaller KL, Caldwell JH. Developmental and regional expression of sodium channel isoform NaCh6 in the rat central nervous system. *J. Comp. Neurol* 2000;420:84–97. [PubMed: 10745221]
- Schaller KL, Caldwell JH. Expression and distribution of voltage-gated sodium channels in the cerebellum. *Cerebellum* 2003;2:2–9. [PubMed: 12882229]
- Schaller KL, Krzemien DM, Yarowsky PJ, Krueger BK, Caldwell JH. A novel, abundant sodium channel expressed in neurons and glia. *J. Neurosci* 1995;15:3231–3242. [PubMed: 7751906]
- Schoorlemmer J, Goldfarb M. Fibroblast growth factor homologous factors and the islet brain-2 scaffold protein regulate activation of a stress-activated protein kinase. *J. Biol. Chem* 2002;277:49111–49119. [PubMed: 12244047]
- Shakkottai VG, Xiao M, Xu L, Wong M, Nerbonne JM, Ornitz DM, Yamada KA. FGF14 regulates the intrinsic excitability of cerebellar Purkinje neurons. *Neurobiol. Dis* 2009;33:81–88. [PubMed: 18930825]
- Smallwood PM, Munoz-Sanjuan I, Tong P, Macke JP, Hendry SH, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. Fibroblast growth factor (FGF) homologous factors: new members of the FGF family implicated in nervous system development. *Proc. Natl. Acad. Sci. USA* 1996;93:9850–9857. [PubMed: 8790420]
- Van Swieten JC, Brusse E, De Graaf BM, Krieger E, Van De Graaf R, De Koning I, Maat-Kievit A, Leegwater P, Dooijes D, Oostra BA, Heutink P. A mutation in the fibroblast growth factor 14 gene is associated with autosomal dominant cerebellar ataxia. *Am. J. Hum. Genet* 2003;72:191–199. [PubMed: 12489043]
- Verhey KJ, Meyer D, Deehan R, Blenis J, Schnapp BJ, Rapoport TA, Margolis B. Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. *J. Cell Biol* 2001;152:959–970. [PubMed: 11238452]
- Wang Q, Bardgett ME, Wong M, Wozniak DF, Lou J, McNeil BD, Chen C, Nardi A, Reid DC, Yamada K, Ornitz DM. Ataxia and paroxysmal dyskinesia in mice lacking axonally transported FGF14. *Neuron* 2002;35:25–38. [PubMed: 12123606]
- Wang Q, McEwen DG, Ornitz DM. Subcellular and developmental expression of alternatively spliced forms of fibroblast growth factor 14. *Mech. Dev* 2000;90:283–287. [PubMed: 10640713]
- Wittmack EK, Rush AM, Craner MJ, Goldfarb M, Waxman SG, Dib-Hajj SD. Fibroblast growth factor homologous factor 2B: association with Nav1.6 and selective colocalization at nodes of ranvier of dorsal root axons. *J. Neurosci* 2004;24:6765–6775. [PubMed: 15282281]

- Wittmack EK, Rush AM, Hudmon A, Waxman SG, Dib-Hajj SD. Voltage-gated sodium channel Nav1.6 is modulated by p38 mitogen-activated protein kinase. *J. Neurosci* 2005;25:6621–6630. [PubMed: 16014723]
- Wozniak DF, Xiao M, Xu L, Yamada KA, Ornitz DM. Impaired spatial learning and defective theta burst induced LTP in mice lacking fibroblast growth factor 14. *Neurobiol. Dis* 2007;26:14–26. [PubMed: 17236779]

Figure 1. FGF14-1b attenuates Nav1.2 currents

Representative whole-cell voltage-gated inward Na^+ currents (I_{Na}) recorded from HEK-Nav1.2 cells transiently expressing GFP or one of FGF14 constructs, as indicated; the voltage-clamp protocol is shown below the current records. Voltage-dependences of I_{Na} steady-state inactivation (**B**) and activation (**C**) were measured as described in *Materials and Methods* and means $\pm$ SEM values are plotted as a function of the membrane potential. The activation and inactivation data were fitted with the Boltzmann function as described in *Materials and Methods*. **D–F**. Bar graphs representing mean $\pm$ SEM peak Nav1.2 current densities (**D**) and the mean $\pm$ SEM voltages of half maximal activation (**E**) and inactivation (**F**), measured in cells expressing GFP (black bar), FGF14-1a (white bar), FGF14-1b (grey bar) or FGF14- Δ NT

(hatched bar). *, ** Values are significantly different from GFP expressing cells at the * $p < 0.05$ or ** $p < 0.01$ level.

Figure 2. FGF14-1B attenuates Nav1.6R currents A

Representative whole-cell voltage-gated inward Na^+ currents (I_{Na}) recorded from ND7/23 cells co-transfected with mouse Nav1.6R and one of the indicated FGF14 constructs; the voltage-clamp protocol is shown below the current records. Voltage-dependences of I_{Na} steady-state inactivation (B) and activation (C) were measured as described in *Materials and Methods* and means $\pm$ SEM values are plotted as a function of the membrane potential. The activation and inactivation data were fitted with the Boltzman function as described in *Materials and Methods*. D–F. Bar graphs representing mean $\pm$ SEM peak Nav1.6R current densities (D) and the mean $\pm$ SEM voltages of half maximal activation (E) and inactivation (F) of Nav1.6R currents, measured in cells expressing GFP (black bar), FGF14-1a (white bar), FGF14-1b (grey

bar) or FGF14-ΔNT (hatched bar). *, ** Values are significantly different from GFP expressing cells at the * $p < 0.05$ or ** $p < 0.01$ level.

Figure 3. FGF14-1a slows recovery from inactivation of Nav1.6R channels

A. Top panel: Double pulse protocol used to examine the kinetics of I_{Na} recovery from inactivation. Lower panel: Mean ($\pm$ SEM) normalized recovery (at -80 mV) from inactivation data measured in cells co-transfected with GFP (black square), FGF14-1a (diamond), FGF14-1b (grey square) and FGF14- Δ NT (triangle) are plotted as a function of recovery time (in seconds). Inset: recovery data for Nav1.6R currents determined in cells co-transfection with GFP, FGF14-1b or FGF14- Δ NT at a smaller time scale. **C.** Mean $\pm$ SEM recovery time constants for Nav1.6R currents in cells co-expressing GFP (black bar), FGF14-1a (white bar), FGF14-1b (grey bar) or FGF14- Δ NT (hatched bar). *Value is significantly ($p < 0.05$) larger than in cells co-expressing GFP.

Figure 4. Marked cumulative inactivation of Nav1.6R currents in cells co-expressing FGF14-1a

A. Mean $\pm$ SEM normalized Nav1.6R current amplitudes, determined as a function of time (test pulse number) after the onset of a repetitive train of depolarizations to -10 mV presented at a frequency of 5 Hz (as described in *Materials and Methods*). In cells expressing Nav1.6R and FGF14-1a a marked attenuation of Nav1.6R currents is evident following the first stimulus (diamond). Nav1.6R currents in cells co-transfected with GFP (black square), FGF14-1b (grey square) or FGF14- Δ NT (triangle), in contrast do not undergo appreciable inactivation at 5 Hz. **B.** The attenuation of mean $\pm$ SEM normalized Nav1.6R current amplitudes varies as a function of the stimulation frequency in cells co-expressing FGF14-1a; values plotted represent the peak currents evoked during the 20th pulse relative to the peak currents evoked by the first pulse of the train at each stimulation frequency. *Values are significantly ($p < 0.05$) different from those measured in cells expressing Nav1.6R and GFP.

Figure 5. FGF14 co-immunoprecipitates with the C-terminal domains of Nav1.2 and Nav1.6
A. Amino acid sequence alignment of Nav1.2 and Nav1.6 C-terminal domains (underlined) tagged with the HA epitope (double underlined). A di-leucine motif (-IL) important for targeting and endocytosis of Nav1.2 and a motif (-PPSY) involved in ubiquitin-mediated degradation of Nav1.2 are also marked (asterisk). **B.** Human HA-Nav1.2 C-tail (CT) or mouse HA-Nav1.6 C-tail (CT) were expressed with FGF14-1b-myc, FGF14-1a-myc, FGF14-ΔNT-myc, or FGF14(F145S)-myc in HEK293 cells. Whole cell lysates were immunoprecipitated with anti-Myc-agarose (IP:Myc) and immunoblots (IB) were performed with either an anti-Myc or an anti-HA antibody.

Figure 6. The FGF14 N-terminal domain controls targeting to the AIS in hippocampal neurons **A, D, G, J.** GFP, FGF14-1a-GFP, FGF14-1b-GFP and FGF14-ΔNT-GFP staining visualized as GFP fluorescence (green). **B, E, H, K.** βIV-spectrin labeling visualized as Alexa350 fluorescence (red). **C, F, I, L.** GFP/βIV-spectrin double-labeling (GFP/Alexa350 co-fluorescence). Note that in contrast to wild-type FGF14-1a-GFP and FGF14-1b-GFP, the FGF14-ΔNT-GFP protein is not targeted to the AIS (compare images **F** and **I** with **L**, and images **D** and **G** with **J**). Insets (**a-l**) are enlargements of the marked regions. **M.** AIS Enrichment Index (fluorescence intensity ratio in the axonal initial segment versus dendrites) was measured in GFP (0.98 ± 0.07 , $n=5$), FGF14-1a-GFP (1.51 ± 0.05 , $n=4$, *** $p < 0.001$ compared to GFP), FGF14-1b-GFP (1.74 ± 0.11 , $n=4$, $p < 0.001$ compared to GFP), and FGF14-

Δ NT-GFP expressing neurons (1.02 ± 0.02 , $n=4$, $p=0.56$ compared to GFP). Scale bars = 10 μ m.

Comparison of voltage-dependent properties of Nav1.2 currents in HEK-Nav1.2 cells co-expressing GFP or FGF14 proteins

Table 1

Condition	Peak Density (pA/pF)	Activation $V_{1/2}$ (mV)	k_{act} (mV)	Inactivation $V_{1/2}$ (mV)	k_{inact} (mV)
GFP	-242 ± 20 (37) [/]	-30 ± 1 (9)	2.7 ± 0.4 (9)	-64 ± 1 (19)	5.7 ± 0.1 (19)
FGF14-1a	-267 ± 39 (10)	-31 ± 4 (4)	4 ± 1 (4)	-55 ± 1 (8)**	3.2 ± 0.4 (8)***
FGF14-1b	-91 ± 11 (28)***	-26 ± 2 (9)*	3.8 ± 0.5 (9)	-64 ± 0.4 (18)	5.8 ± 0.2 (18)
FGF14-ΔNT	-251 ± 4 (20)	-30 ± 0.5 (14)	-3.8 ± 0.4 (14)	-69 ± 1 (18)****	-6.2 ± 0.4 (18)

[/] All values are mean ± SEM; numbers in parentheses are the numbers of cells analyzed.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$ levels different from those determined in cells expressing GFP alone.

Comparison of voltage-dependent properties of Nav1.6 in ND7/23 cells co-expressing GFP or FGF14 proteins

Table 2

Condition	Peak Density (pA/pF)	Activation $V_{1/2}$ (mV)	k_{act} (mV)	Inactivation $V_{1/2}$ (mV)	k_{inact} (mV)
GFP	74 ± 8 (30)	-19.3 ± 0.8 (28) [/]	7.0 ± 0.2 (28)	-68.0 ± 0.7 (25)	6.9 ± 0.3 (25)
FGF14-1a	81 ± 24 (12)	-19.2 ± 1.1 (12)	7.9 ± 0.4 (12)	-58.1 ± 1.3 (8) [*]	5.8 ± 0.5 (8)
FGF14-1b	14 ± 3 (15) ^{**}	-18.6 ± 1.2 (9)	8.2 ± 0.5 (9) [*]	-54.4 ± 1.7 (10) [*]	6.6 ± 0.5 (10)
FGF14-ΔNT	168 ± 20 (12) ^{***}	-15.2 ± 1.2 (11) [*]	7.0 ± 0.2 (11)	-63.8 ± 1.4 (10) [*]	5.2 ± 0.2 (10) [*]

[/] All values are mean ± SEM; numbers in parentheses are the numbers of cells analyzed.

^{*} p < 0.05

^{**} p < 0.01

^{***} p < 0.001 levels different from those determined in cells expressing GFP alone.

Table 3

Relative sodium current at the 20th pulse in train stimulation of cells co-transfected with Nav1.6 and FGF14 proteins

Frequency (Hz)	Relative Current at the 20 th Pulse			
	GFP	FGF14-1a	FGF14-1b	FGF14-ΔNT
0.5	0.99 ± 0.01 (18)	0.93 ± 0.03 (6)	1.03 ± 0.07 (5)	1.01 ± 0.01 (11)
1	1.01 ± 0.01 (21)	0.87 ± 0.01 (10)*	0.98 ± 0.71 (5)	1.00 ± 0.01 (11)
2	0.98 ± 0.01 (21)	0.68 ± 0.02 (10)*	1.01 ± 0.06 (5)	0.99 ± 0.01 (11)
5	0.97 ± 0.01 (21)	0.33 ± 0.03 (10)*	1.01 ± 0.02 (5)*	0.97 ± 0.01 (11)
10	0.93 ± 0.01 (23)	0.17 ± 0.02 (10)*	0.96 ± 0.02 (7)	0.96 ± 0.01 (11)

¹All values are mean ± SEM; numbers in parentheses are the numbers of cells analyzed.

* p < 0.05 levels different from those determined in cells expressing GFP alone.