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FGF14 Localization and Organization of the Axon Initial Segment

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Abstract

The axon initial segment (AIS) is highly enriched in the structural proteins ankyrin G and IV-spectrin, the pore-forming () subunits of voltage-gated sodium (Nav) channels, and functional Nav channels, and is critical for the initiation of action potentials. We previously reported that FGF14, a member of the intracellular FGF (iFGF) sub-family, is expressed in cerebellar Purkinje neurons and that the targeted inactivation of *Fgf14* in mice (*Fgf14*^{−/−}) results in markedly reduced Purkinje neuron excitability. Here, we demonstrate that FGF14 immunoreactivity is high in the AIS of Purkinje neurons and is distributed in a decreasing, proximal to distal, gradient. This pattern is evident early in the postnatal development of Purkinje neurons and is also observed in many other types of central neurons. In (*Scn8a*^{med}) mice, which are deficient in expression of the Nav1.6 subunit, FGF14 immunoreactivity is markedly increased and expanded in the Purkinje neuron AIS, in parallel with increased expression of the Nav1.1 (*Scn1a*) subunit and expanded expression of IV-spectrin. Although Nav1.1, FGF14, and IV-spectrin are affected, ankyrin G immunoreactivity at the AIS of *Scn8a*^{med} and wild type (WT) Purkinje neurons was not significantly different. In *Fgf14*^{−/−} Purkinje neurons, IV-spectrin and ankyrin G immunoreactivity at the AIS were also similar to WT Purkinje neurons, although both the Nav1.1 and Nav1.6 subunits are modestly, but significantly ($P < 0.005$), reduced within sub-domains of the AIS, changes that may contribute to the reduced excitability of *Fgf14*^{−/−} Purkinje neurons.

Keywords

Purkinje neuron; axon initial segment; AIS; iFGF; FGF14; voltage-gated sodium channel; Nav1.1; *Scn1a*; Nav1.6; *Scn8a*; ankyrin G; IV-spectrin

Introduction

The axon initial segment (AIS) is a specialized structural and functional region in neurons that is critical for the generation of action potentials. The AIS is delineated by the scaffolding protein, ankyrin G (AnkG) (Zhou et al., 1998), the cytoskeletal adaptor protein, IV-spectrin (Yang et al., 2007), and the cell adhesion molecules, neurofascin-186 (NF-186) (Hedstrom et al., 2007) and neuronal cell adhesion molecule (NrcAM) (Davis et al., 1996). In addition, the AIS is highly enriched in voltage-gated Na⁺ (Nav) channels. In the absence of ankyrin G or IV-spectrin, the localization of Nav channel pore-forming (a) subunit to the

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AIS is disrupted, action potential generation is impaired, and neuronal excitability is reduced (Komada and Soriano, 2002; Winkels et al., 2009; Zhou et al., 1998).

In the central nervous system, three different Nav subunits, Nav1.1 (*Scn1a*), Nav1.2 (*Scn2a1*), and Nav1.6 (*Scn8a*), are enriched in the AIS (Hu et al., 2009; Kole et al., 2008; Stuart and Sakmann, 1994). Interestingly, these Nav subunits are differentially expressed in different types of CNS neurons and display distinct distribution patterns within the AIS, differences that contribute to cell-type specific firing properties (Lorincz and Nusser, 2008). Nav1.1, for example, is restricted to a small proximal subregion of the AIS of GABAergic inhibitory neurons (Ogiwara et al., 2007) and retinal ganglion cells (Van Wart et al., 2007) and displays little overlap with Nav1.6, which localizes to the distal AIS. Purkinje neurons also express Nav1.1 or Nav1.6, and mice lacking either Nav1.1 or Nav1.6 show defects in firing, suggesting that both Nav subunits are required for normal Purkinje neuron function (Kalume et al., 2007; Raman et al., 1997; Vega-Saenz de Miera et al., 1997). Deletion of *Scn1a* (Nav1.1) (Kalume et al., 2007; Yu et al., 2006) or *Scn8a* (Nav1.6) (Meisler et al., 2004) result in seizures, ataxia, impaired motor function, and early death, revealing that the important physiological roles of Nav channels encoded by these subunits regulate neuronal activity in multiple regions of the central nervous system.

Intracellular FGFs (iFGFs 11–14), also called Fibroblast Growth Factor Homologous Factors (FHF), are distinct from canonical FGFs in that the iFGFs are not secreted and do not interact with classical tyrosine kinase FGF receptors (Itoh and Ornitz, 2011). FGF14 and the other iFGFs are widely expressed throughout the nervous system (Smallwood et al., 1996; Wang et al., 2000; Xiao et al., 2007) and are concentrated at the AIS of cultured hippocampal neurons (Grubb and Burrone, 2010; Laezza et al., 2007) and in nodes of Ranvier (Wittmack et al., 2004). FGF14 interacts directly with the C-termini of Nav subunits, including Nav1.1, Nav1.2, Nav1.6, in HEK293 cells (Laezza et al., 2007; Laezza et al., 2009; Lou et al., 2005), and the targeted deletion of *Fgf14* (FGF14) results in an ataxia-like phenotype (Wang et al., 2002) and markedly attenuated excitability in cerebellar Purkinje (Shakkottai et al., 2009; Xiao et al., 2007) and granule (Goldfarb et al., 2007) neurons. Indeed, the phenotypic consequences of the loss of FGF14 are similar to those resulting from loss of function mutations in Nav1.1 and Nav1.6 (Kalume et al., 2007; Meisler et al., 2004; Yu et al., 2006). Interestingly, mutations in *FGF14* have been linked to spinocerebellar ataxia (SCA27) in humans (Brusse et al., 2006; Dalski et al., 2005; Misceo et al., 2009; Van Swieten et al., 2003; Wang et al., 2002).

Here, we show that FGF14 is preferentially localized in a decreasing proximal to distal gradient along the AIS of adult Purkinje neurons, as well as in other type of central neurons. This pattern is evident early in the postnatal development of Purkinje neurons *in vivo* and is recapitulated in isolated Purkinje neurons maintained *in vitro*. Additional experiments demonstrate that the intensity of FGF14 and Nav1.1 immunostaining are increased along the AIS of Purkinje neurons in *Scn8a^{med}* mice, which lack Nav1.6, whereas the intensity of IV-spectrin and ankyrin G at the AIS of *Scn8a^{med}* Purkinje neurons is similar to WT Purkinje neurons. In addition, although the AIS scaffolding proteins, IV-spectrin and ankyrin G, are not significantly affected by the loss of FGF14, Nav1.1 and Nav1.6 immunostaining is decreased in *Fgf14^{-/-}* Purkinje neurons, changes that may contribute to the reduced excitability of these cells (Shakkottai et al., 2009).

Materials and Methods

Mice

Fgf14^{-/-} mice were generated as previously described and maintained on an inbred C57BL/6J background (Wang et al., 2002). All genotypes were confirmed by PCR analysis (Wang

et al., 2002). Heterozygous male and female *Scn8a^{med}* (Burgess et al., 1995) mice (C3Fe.Cg-*Scn8a^{med}*/J, JAX stock number 003798) were purchased from the Jackson Lab. *Scn8a^{med}* mice were bred with wild type (WT) C57BL/6J mice. Genotypes were determined by PCR (forward primer 5'-GGAGCAAGGTTCTAGGCAGCTTTAAGTGTG, reverse primer 5'-GGACTTAGAATGTACAAGGCAGGAG, WT band 280 bp, mutant band 460 bp) as described previously (Kohrman et al., 1996; Van Wart and Matthews, 2006). Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals (NIH). All protocols involving animals were approved by the Animal Studies Committee at Washington University Medical School.

Primary cerebellar Purkinje neuron cultures

Dissociated cerebellar cultures were prepared as described previously (Tabata et al., 2000) with some modifications. Briefly, the cerebellum was dissected and the meninges were removed (on ice) from individual postnatal day 0–1 (P0/1) *Fgf14^{+/-}* mouse pups produced by crossing heterozygous *Fgf14^{+/-}* mice. Dissected tissue from each animal was washed twice with ice-cold Ca^{2+} and Mg^{2+} -free HBSS (Invitrogen) and incubated in papain (papain dissociation kit system for neural cells, Worthington Biochemical Corporation) for 23 min at 37°C in a shaking water bath. Digestion was terminated by addition of albumin-ovomucoid inhibitor solution, followed by two washes in DMEM/F-12 (1:1) medium (Invitrogen). Tissues were triturated gently using fire-polished Pasteur pipettes and the resulting cell suspensions were centrifuged at 300 g for 5 min at room temperature. Cell pellets were resuspended in media, centrifuged at 100 g for 5 min, resuspended in seeding medium (10% FBS in DMEM/F-12), and plated onto 0.01% poly-D-lysine-coated coverslips (12mm², Fisher Scientific) in 24 well plates at a density of 5×10^6 cells/ml. Approximately 24 hr later, Purkinje neuron culture media (100 μM putrescine, 30 nM sodium selenite, 1.4 mM L-glutamine, 20 nM progesterone, 10 ng/ml insulin, 100 mg/ml transferrin, 0.5 ng/ml tri-iodotyronine, 10 $\mu\text{g}/\text{ml}$ gentamicin in 1:1 DMEM/F-12) was added to each well. Half of the culture medium was replaced with fresh medium containing 4 μM cytosine arabinoside (AraC) (to inhibit glial proliferation) after 6 and 12 days *in vitro* (DIV).

Tissue preparation and immunostaining

Mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) or ketamine-atropine-xylazine (KAX, 30 mg/ml ketamine, 0.2 mg/ml atropine, and 4 mg/ml xylazine in 1.0 ml/kg, i.p.) and transcardially perfused with a vascular rinse of 0.9% NaCl followed by ice-cold 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were dissected, postfixed in the same solution overnight at 4°C, and cryoprotected in 30% sucrose, 0.1 M phosphate buffer until they sank. After embedding in O.C.T. compound, brains were cryosectioned (sagittal) at 14 μm or 30 μm and collected on positive charged slides. For immunohistochemistry, brain sections were washed in PBS 3X, blocked in a solution of 7.5% goat serum (Sigma) and 0.25% Triton X-100 (TX-100; Sigma) in PBS. Sections were then incubated at 4°C overnight with the primary antibodies diluted in 1% bovine serum albumin (BSA), 0.25% TX-100 in PBS. Sections were then washed 3X for 5 min at room temperature in PBS before application of secondary antibodies. Sections were then incubated in the appropriate secondary goat antibodies (labeled with Alexa 488, 594 or 647) against mouse, rabbit or guinea pig antibodies at 1:250 dilution in the same solution as used for the primary antibodies (Molecular Probes).

The following primary antibodies were used: mouse monoclonal anti-panNav (1:1000, Sigma); mouse monoclonal anti-Nav1.1 (1:1000, NeuroMab); rabbit polyclonal anti-Nav1.1 (1:250, Sigma); mouse monoclonal anti-Nav1.6 (1:1000, NeuroMab); rabbit polyclonal anti-Nav1.6 (1:500, Alomone) and mouse monoclonal anti-FGF14 (1:1000, NeuroMab); mouse monoclonal anti-ankyrin G (1:1000, gift from P. Mohler or NeuroMab); rabbit polyclonal

anti-ankyrin G (1:500, gift from P. Mohler or Santa Cruz); chick polyclonal anti-IV-spectrin (1:1000, gift from M. Komada); mouse monoclonal anti-calbindin D (Calb1) (1:20,000, Swant); rabbit polyclonal anti-calbindin 1 (D-28K; 1:20,000; Swant); guinea pig polyclonal anti-calbindin 1 (D-28K; 1:2000; Synaptic Systems).

Western blotting

Mice were sacrificed by CO₂ asphyxiation, decapitated, and cerebella were removed and placed in ice-cold brain homogenization buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (1 mM PMSF and Calbiochem, set #3). Cerebella were homogenized on ice in a Dounce homogenizer. Homogenates were rotated for 15 min at 4°C and then centrifuged at 3000 rpm for 10 min at 4°C to pellet nuclei. Protein concentrations were measured using a BCA assay (Pierce). Proteins were electrophoresed on 4–15% polyacrylamide gels (BioRad). Resolved proteins were transferred to PVDF membranes (Millipore) for 1.5 h at 4°C and blocked in tris-buffered saline (TBS) with 3% nonfat dry milk and 0.1% Tween-20. Membranes were incubated in primary antibodies diluted in TBS with 0.5% nonfat dry milk and 0.1% Tween-20.

The following primary antibodies were used: mouse monoclonal anti-panNav (1:1000, Sigma); mouse monoclonal anti-Nav1.1 (1:1000, NeuroMab); rabbit polyclonal anti-Nav1.6 (1:300, gift from J. Trimmer); and rabbit polyclonal anti-FGF14 (1:1000). Rabbit polyclonal serum against FGF14 was made by inoculating rabbits with an FGF14 peptide (Lou et al., 2005). Washed membranes were incubated with goat-anti-mouse-HRP or goat-anti-rabbit-HRP (1:5000, Santa-Cruz) and visualized with the Super Signal Femto chemiluminescence substrate (Pierce).

Imaging

All imaging was performed on a Zeiss Apotome microscope fitted with an AxioCam digital camera (Carl Zeiss). AxioVision software was used for collection of fluorescence images. Comparison of WT and *Fgf14*^{-/-} or WT and *Scn8a*^{med} tissue was performed on slices prepared in parallel and images were acquired using identical exposure times. All images were collected by Z-scan with 0.5 μm steps. Fluorescence intensity (FI) was measured using NIH ImageJ software. The contrast and brightness of displayed images were adjusted using Canvas X software. Image stacks for double immunostained slides were converted into the same single average intensity Z-axis projection and exported as TIFF files. Fluorescence intensities for FGF14, Nav1.1 and Nav1.6 double stained with ankyrin G or IV-spectrin were measured using the NIH ImageJ line-scan module starting within the soma and extending through the length of the AIS, as defined by the pattern of ankyrin G or IV-spectrin immunostaining. Quantification of the lengths and fluorescence intensities of the AIS staining was performed using a previously described method (Grubb and Burrone, 2010). Briefly, the start and end positions of the AIS were defined as 33% of the maximum fluorescence intensity of ankyrin G or IV-spectrin. The background (for ankyrin G, IV-spectrin or FGF14) was subtracted based on a 1 μm segment within the soma along the vector of the AIS. For Nav1.1 and Nav1.6 immunostaining, the background was subtracted based on a 1 μm segment distal to the AIS. AIS fluorescence density was defined as the sum of AIS fluorescence intensity divided by AIS length.

Statistical analysis

Two-tailed Student's t tests were performed where appropriate. All the results are presented as mean ± SEM as indicated.

Results

FGF14 is localized in a proximal to distal gradient along AIS in CNS neurons

To examine the distribution and localization of FGF14 *in situ* in the adult CNS, we used an anti-FGF14 monoclonal antibody on cryostat sections prepared from adult WT (*Fgf14^{+/+}*) and *Fgf14^{-/-}* (Wang et al., 2002) mice (Figure 1). *In situ* immunoreactivity for FGF14 was detected in the AIS of neurons in several regions of the *Fgf14^{+/+}* CNS, including Purkinje neurons, hippocampal CA1 pyramidal neurons, and neurons in layers II to VI of the motor cortex (Figure 1A, D, F). In the cerebellum, FGF14 immunostaining was also observed at the AIS of granule and basket neurons (Figure 1A). No AIS FGF14 immunoreactivity, however, was detected in sections from *Fgf14^{-/-}* mice (Figure 1A, lower panels), validating the specificity of the anti-FGF14 antibody. Quantitative analysis of the intensity of the FGF14 immunolabeling along the length of the AIS revealed higher expression proximally and lower expression distally (Figure 1A, B). In contrast, the intensity of the anti-IV-spectrin staining appeared to be uniform along the AIS. Graded expression of FGF14 was also observed in the AIS of hippocampal CA1 pyramidal neurons (Figure 1D,E), as well as in neurons in layers II to VI of the motor cortex (Figure 1F, G). Although less intense, FGF14 immunostaining was also evident on Purkinje neuron somata in WT, but not in *Fgf14^{-/-}* mice (Figure 1A inset).

Anti-FGF14 staining was also examined in WT cerebellar Purkinje neurons isolated at postnatal day 0–1 and allowed to mature *in vitro* for approximately two weeks. In culture, Purkinje neurons were identified by their large somata and by the presence of the Ca²⁺ binding protein, calbindin1 (Figure 1H, I) (Jenkins and Bennett, 2001). Labeling of these cells with the anti-FGF14 antibody also revealed a decreasing proximal to distal gradient of FGF14 immunostaining along the AIS, whereas IV-spectrin immunofluorescence intensity was uniform along the AIS, but was not observed at the axon hillock (Figure 1H, I). Interestingly, FGF14 immunoreactivity was evident in the axon hillock, proximal to detectable anti-IV-spectrin labeling (Figure 1I, arrows). Taken together, these data reveal that, *in vivo* and *in vitro*, endogenous FGF14 is localized in a decreasing proximal to distal gradient along the AIS in Purkinje and other CNS neurons.

FGF14 is not required for assembly of the AIS during Purkinje neuron development

To explore the possible relationship(s) between FGF14 expression/localization and assembly of the AIS, we examined labeling with anti-FGF14, anti-ankyrin G, and anti-IV-spectrin antibodies at different times during *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neuron maturation *in situ*. Similar to the adult (Figure 1), immature Purkinje neurons were readily identified by immunostaining for calbindin 1 (data not shown) as early as postnatal day 1–2. As illustrated in Figure 2, weak staining with the anti-FGF14 (Figure 2A), anti-IV-spectrin (Figure 2A), and anti-ankyrin G (Figure 2B,C) antibodies was detected in the AIS of *Fgf14^{+/+}* Purkinje neurons as early as postnatal day 1/2. At this stage, FGF14 immunofluorescence appears to be uniformly distributed along the length of the AIS (Figure 2A). In contrast, at postnatal days 8/9 and 14/15, FGF14 immunoreactivity was decidedly less uniform, similar to that of adult Purkinje neurons (Figure 1), where anti-FGF14 staining was higher in the proximal and lower in the distal AIS. Similar to postnatal day 1/2, however, anti-ankyrin G (Figure 2B, C) and anti-IV-spectrin immunostaining (data not shown) appeared to be uniformly distributed along the length of the AIS at postnatal days 8/9 and 14/15. In addition, the pattern of anti-ankyrin G immunostaining at the AIS of *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neurons at postnatal days 2/3, 8/9 and 14/15 (Figure 2B,C) was similar.

In early postnatal (day 2/3) cerebellum, faint labeling with the anti-Nav1.6- and anti-Nav1.1-specific antibodies was also detectable in Purkinje neuron AIS (Figure 2B,C), although no

clear staining patterns were resolved. The distinct patterns of anti-Nav1.1 and anti-Nav1.6 immunofluorescence that are evident in the AIS of many mature neuronal cell types (Duflocq et al., 2008; Lorincz and Nusser, 2008; Ogiwara et al., 2007; Van Wart et al., 2007), including mature Purkinje neurons (see also Figure 6), become apparent in early postnatal (days 8/9 and 14/15) *Fgf14*^{+/+} Purkinje neurons: Nav1.6 immunoreactivity was evident in a proximal (low) to distal (high) gradient in the AIS (Figure 2B), whereas Nav1.1 immunoreactivity appeared restricted to the proximal third of the AIS (Figure 2C), in *Fgf14*^{+/+} Purkinje neurons. The adult patterns of Nav1.1 and Nav1.6 subunit localization in the AIS, therefore, begin to emerge early during the developmental maturation of Purkinje neurons *in vivo*. Interestingly, the patterns of anti-Nav1.1 and anti-Nav1.6 immunoreactivity at the AIS of *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neurons at postnatal days 2/3, 8/9 and 14/15 (Figure 2B,C) were qualitatively similar, although it does appear that Nav1.1 immunofluorescence was expanded along the AIS of *Fgf14*^{-/-}, compared with *Fgf14*^{+/+}, Purkinje neurons (see blue arrows in Figure 2C), suggesting a relationship between FGF14 and Nav1.1 expression and/or localization.

FGF14 and Nav1.1 are increased in neurons lacking Nav1.6

It has previously been reported that Purkinje neuron spontaneous firing is impaired in (*Scn8a*^{med}) mice lacking Nav1.6 (Khaliq et al., 2003) and, in addition, that Nav1.1 immunoreactivity is expanded along the length of the AIS in *Scn8a*^{med} Purkinje neurons (Van Wart and Matthews, 2006). To determine if the distribution of FGF14 is also affected by the loss of Nav1.6 and the alterations in Nav1.1, we compared the relative levels and the patterns of anti-FGF14 and anti-Nav1.1 labeling at the AIS of Purkinje neurons in *Scn8a*^{med} and WT mice. These experiments revealed that the pattern of anti-FGF14 immunostaining was similar in the AIS of WT and *Scn8a*^{med} Purkinje neurons, with higher levels in the proximal and lower levels in the distal AIS (Figure 3A). Quantitative linescan analysis, however, revealed that the intensity of anti-FGF14 immunostaining was significantly ($p < 0.01$) higher throughout the length of the AIS of *Scn8a*^{med}, compared with WT, Purkinje neurons and extended more distally (Figure 3B, Table 1). Analysis of IV-spectrin immunofluorescence revealed no significant differences in staining intensity in WT and *Scn8a*^{med} Purkinje neurons, although anti-IV-spectrin labeling was increased distally along the axons of *Scn8a*^{med}, compared with WT, Purkinje neurons (Figure 3C, Table 1), suggesting expansion of the AIS (see Discussion).

Consistent with previously published observations (Van Wart and Matthews, 2006), anti-Nav1.1 immunofluorescence intensity was increased in the proximal AIS and expanded distally along the length of *Scn8a*^{med} Purkinje neuron axons, whereas ankyrin G immunofluorescence intensity was decreased (Figure 3D–F, Table 1). Comparison of Nav1.1 and FGF14 distributions in *Scn8a*^{med} Purkinje neurons revealed similar increases in both (Table 1). Although anti-FGF14 and anti-Nav1.1 immunostaining were increased in *Scn8a*^{med} animals in parallel with the loss of Nav1.6, Western blot analysis (Figure 4) revealed that the total protein expression levels of FGF14 and Nav1.1 are qualitatively not different in WT and *Scn8a*^{med} cerebellar lysates.

Remodeling of Nav channel α subunits at the AIS in *Fgf14*^{-/-} Purkinje neurons

The observation that FGF14 immunofluorescence is expanded along the AIS of *Scn8a*^{med} Purkinje neurons in parallel with the increase in anti-Nav1.1 immunoreactivity (Figure 3) suggests that FGF14 and Nav1.1 may be coordinately regulated. The observation that Nav1.1 labeling appears to be expanded along the AIS of developing *Fgf14*^{-/-}, compared with *Fgf14*^{+/+}, Purkinje neurons (see blue arrows in Figure 2C) also suggests a relationship between the localization of the FGF14 and Nav1.1 proteins. To explore this hypothesis further, we examined the distributions of the AIS scaffold proteins, ankyrin G and IV-

spectrin, and of the Nav1.1 and Nav1.6 subunits at the AIS of adult *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neurons.

As illustrated in Figure 5, anti-ankyrin G and anti-IV-spectrin immunoreactivity were colocalized at the AIS of adult *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neurons (Figure 5A). Quantitative linescan analysis revealed no differences in the overall length of the AIS or in the intensity of anti-ankyrin G immunofluorescence in the AIS of *Fgf14^{-/-}*, compared to *Fgf14^{+/+}*, Purkinje neurons (Figure 5A,B, Table 2). Parallel analyses revealed that IV-spectrin immunofluorescence intensity was modestly lower (by ~30%) in *Fgf14^{-/-}*, compared with *Fgf14^{+/+}*, Purkinje neuron AIS (Figure 5A,C Table 2). The distribution patterns and the intensities of anti-ankyrin G and anti-IV-spectrin labeling were also similar in *Fgf14^{-/-}* and *Fgf14^{+/+}* cortical (layers II to VI) neurons and in hippocampal CA1 pyramidal neurons (data not shown). FGF14, therefore, appears not to be required for the maintenance of the AIS in mature (Purkinje or other central) neurons.

Experiments were also completed to examine the localization and relative distribution levels of Nav1.1 and Nav1.6 at the AIS of mature *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neurons (Figure 6). Linescan analysis revealed increased anti-Nav1.6 immunofluorescence intensity over most of the length of the AIS in *Fgf14^{+/+}* Purkinje neurons (Figure 6A,C), consistent with a proximal to distal gradient in Nav1.6. In contrast, anti-Nav1.1 immunoreactivity at the AIS was highest proximal to the cell body (Figure 6A). Anti-Nav1.6 and anti-Nav1.1 immunofluorescence intensities were decreased at the AIS of *Fgf14^{-/-}*, compared with *Fgf14^{+/+}*, Purkinje neurons (Figure 6A). Western blot analyses, however, revealed no differences in the (total) expression levels of the Nav1.1 and Nav1.6 proteins in *Fgf14^{+/+}* and *Fgf14^{-/-}* cerebellar lysates (Figure 7), suggesting that the apparent differences in anti-Nav1.6 and anti-Nav1.1 immunoreactivity at the AIS of *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neurons (Figure 6A) reflect changes in the distribution/localization of Nav subunits in parallel with the loss of FGF14.

To explore this hypothesis, additional immunofluorescence experiments were completed to quantify anti-Nav1.1 and anti-Nav1.6 immunoreactivities in the AIS of individual *Fgf14^{+/+}* and *Fgf14^{-/-}* Purkinje neurons. In these experiments, cerebellar sections were double labeled with antibodies to ankyrin G and Nav1.1 or to ankyrin G and Nav1.6 (Figure 6B–D) and anti-Nav1.1, anti-Nav1.6, and anti-ankyrin G staining intensities, were measured along the AIS. In *Fgf14^{-/-}* Purkinje neurons, mean $\pm$ SEM anti-Nav1.6 immunofluorescence intensity was lower throughout the length of the AIS than in *Fgf14^{+/+}* Purkinje neurons (Figure 6B,C, Table 2). Linescan analysis of anti-Nav1.1 immunofluorescence in *Fgf14^{-/-}* Purkinje neurons also revealed reduced mean $\pm$ SEM Nav1.1 labeling throughout the AIS, which was most prominent in the proximal 6 μ m of the AIS (Figure 6D,E, Table 2). For further quantification, anti-Nav1.1 and anti-Nav1.6 labeling intensities along the AIS in individual Purkinje neurons were measured and normalized to the anti-ankyrin G immunofluorescence staining intensity at the same location in the same cell. As illustrated in Figure 6C, the mean $\pm$ SEM anti-Nav1.6/anti-ankyrin G relative fluorescence intensity along the middle 6–12 μ m of the AIS was significantly ($p < 0.05$) lower in *Fgf14^{-/-}*, compared with *Fgf14^{+/+}*, Purkinje neurons. Plotting the mean $\pm$ SEM anti-Nav1.1/anti-ankyrin G relative fluorescence intensity ratios as a function of distance along the AIS revealed a significant ($p < 0.05$) decrease in the Nav1.1/ankyrin G fluorescence intensity ratio in *Fgf14^{-/-}*, compared with *Fgf14^{+/+}*, Purkinje neurons particularly along the first ~4 μ m of the AIS (Figure 6E, Table 2). Taken together, these analyses suggest that FGF14, which is localized in a proximal (high) to distal (low) gradient along the AIS, is required to attain/maintain high proximal Nav1.1 expression and maximal Nav1.6 expression over the length of the AIS.

Discussion

Here, we demonstrate that FGF14 is prominently expressed in a unique, proximal to distal, gradient along the AIS of mature cerebellar Purkinje neurons and that this localization pattern develops early during the postnatal development of these cells. A similar pattern of AIS labeling was seen in mature hippocampal and cortical neurons. Additional data are presented here demonstrating that FGF14 and Nav1.1 immunostaining were increased and expanded distally along the AIS of Purkinje neurons in mice lacking Nav1.6 (*Scn5a^{med}*), suggesting a regulatory interaction(s) between these proteins. Conversely, anti-Nav1.1 and anti-Nav1.6 immunolabeling were both decreased and anti-Nav1.1 staining was expanded along the AIS of *Fgf14^{-/-}* Purkinje neurons, suggesting a role for FGF14 in regulating the densities and distributions of Nav1.1-encoded and Nav1.6-encoded Nav channels in the AIS of mature Purkinje neurons. Through regulation of Nav subunit expression and patterning, FGF14 and other iFGFs may modulate the firing properties of Purkinje (and other types of central) neurons.

Recent studies have revealed diverse AIS expression patterns of Nav subunits in different neuronal types (Lorincz and Nusser, 2008), suggesting that cell type-specific mechanisms are operative that organize and maintain AIS Nav subunit composition, resulting in the generation of specific physiological properties (Clark et al., 2009). The major Nav subunit, Nav1.6, for example, is expressed alone or with Nav1.1/Nav1.2 in different neurons and in different sub-domains of the AIS (Duflocq et al., 2008; Hu et al., 2009; Lorincz and Nusser, 2008). Complementary expression patterns of Nav1.1 and Nav1.6 have been identified in the AIS of cortical and cerebellar interneurons, as well as cerebellar Purkinje neurons, spinal cord neurons, and retinal ganglion cells (Boiko et al., 2003; Duflocq et al., 2008; Ogiwara et al., 2007; Van Wart and Matthews, 2006). In each of these neuronal cell types, Nav1.1 is preferentially localized in the very proximal AIS and Nav1.6 is distributed more uniformly along the length of the AIS, distal to Nav1.1.

The cellular and molecular mechanisms that regulate the complex patterns of Nav subunit localization in the AIS are likely to be complex. For Nav subunits distributed at high levels proximally and low levels distally, for example, the amount of available Nav subunit protein could be limiting and, once directed towards the axonal compartment, available protein could be sequestered at the proximal AIS or, alternatively, degraded distally. In contrast, for Nav subunits distributed at low levels proximally and at higher levels distally, there may be mechanisms that limit the affinities of these subunits for AIS scaffolding proteins in the proximal, compared to the distal, AIS, perhaps by competition with higher affinity proximally localized Nav subunits for scaffold proteins. In this model, the molecules that function to effect the differential organization of the AIS are themselves also likely to be asymmetrically localized along the length of the axon. One such molecule is neurofascin (NF186), which is an extracellular matrix protein that is localized at high levels proximally and low levels distally (Ango et al., 2004). It is not known, however, whether the distribution of neurofascin affects the localization of Nav subunits in the AIS.

In contrast with the differential AIS localization of the Nav1.1 and Nav1.6 subunits, the ankyrin G and IV-spectrin scaffolding proteins appear to be uniform along the AIS of Purkinje neurons. Although ankyrin G and IV-spectrin are required for Nav subunit localization in the AIS, it is unlikely that either directly regulates the observed localization patterns of the Nav1.1 and/or Nav1.6 subunits. The intracellular location (Itoh and Ornitz, 2011; Smallwood et al., 1996; Wang et al., 2000; Xiao et al., 2007), ability to interact directly with the C termini of Nav subunits (Wang et al., 2011), and the graded AIS distribution (present study), suggest possible roles for FGF14 in modulating the localization of Nav subunits in Purkinje neuron AIS. Clearly, however, FGF14 cannot be the sole

determinant of the pattern(s) of Nav subunit localization because the AIS distribution of FGF14 is perturbed in response to changes Nav subunit expression, as observed in *Scn8a^{med}* Purkinje neurons, which lack Nav1.6 and have increased FGF14 (and Nav1.1).

In mice lacking FGF14, the overall level of Nav1.6 was decreased along the length of the Purkinje neuron AIS. In contrast, the intense proximal localization of Nav1.1 is lost *Fgf14^{-/-}* Purkinje neurons, and weak anti-Nav1.1 immunostaining was evident distally suggesting expansion of the Nav1.1, although the overall amount of Nav1.1 protein within the AIS may not be changed. The distribution patterns of the scaffolding proteins, ankyrin G and IV-spectrin, in contrast, appear not to be affected by the absence of FGF14, although there was a modest decrease in the intensity of anti-IV-spectrin immunoreactivity. These observations suggest that FGF14 is not required to initiate or maintain the structural components of the AIS. We hypothesize that FGF14 may function to regulate the affinity of Nav subunits to scaffolding proteins in the AIS or to other intermediary proteins that could couple Nav subunits to the protein components of the AIS scaffold. These scaffolding proteins include the intracellular proteins ankyrin G and IV-spectrin, as well as the extracellular cell adhesion molecules neurofascin-186 and neuron glia-related cell adhesion molecule, NrCAM (Jenkins and Bennett, 2001; Zhou et al., 1998).

There are four members of the iFGF subfamily and these proteins display distinct abilities to interact with the carboxyl terminal tails of different Nav subunits. For example, FGF14 interacts differentially with the carboxyl terminal tails of Nav1.1, 1.2, 1.5 and 1.6 (Laezza et al., 2009; Lou et al., 2005). Similarly, FGF13 does, whereas FGF12 does not, interact with the carboxyl terminal tail of Nav1.1, whereas both FGF12 and FGF13 interact with the carboxyl terminal tails of other Nav subunits (Wang et al., 2011). Differential interactions between iFGFs and Nav subunits may contribute to the ability of the iFGFs to regulate the distributions of Nav subunits in the AIS. Although the affinity of FGF14 for either the Nav1.1 or the Nav1.6 subunit has not been determined directly, FGF14 and the carboxyl terminus of Nav1.1 are efficiently co-immunoprecipitated, suggesting a relatively high affinity interaction (Lou et al., 2005). Interestingly, there were increased levels of FGF14 and Nav1.1 in the AIS of Purkinje neurons in *Scn8a^{med}* mice, consistent with a model (Figure 8) in which the distributions of FGF14 and Nav1.1 are coordinately regulated. In the absence of FGF14, all AIS Nav subunit levels were reduced, suggesting that FGF14 may be limiting. Consistent with this suggestion, overexpression of FGF14 in hippocampal neurons resulted in increased levels of Nav subunits in the AIS (Laezza et al., 2007; Lou et al., 2005). These observations suggest a model in which limiting amounts of FGF14, different FGF14-Nav subunit binding affinities, competition among the Nav subunits for FGF14 binding and, ultimately, the ability of FGF14 to regulate interactions between/among individual Nav subunits and AIS scaffolding proteins, combine to determine the variable distributions of Nav subunits along the AIS.

The contributions of other members of the iFGF family to AIS organization are not known. Functional redundancy between FGF12 and FGF14 (FHF1 and FHF4) in cerebellar granule cells *in vivo*, however, has been demonstrated (Goldfarb et al., 2007). It has also been demonstrated that FGF14 can form homodimers (Laezza et al., 2007), as well as heterodimers with other iFGFs (MKB., unpublished data) *in vitro*, suggesting that complex combinatorial interactions among iFGFs may further regulate the functioning, as well, perhaps, as the patterning of Nav subunit localization in the AIS.

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Abbreviations

AIS	axon initial segment
FGF14	fibroblast growth factor 14
iFGF	intracellular fibroblast growth factor
Nav	voltage gated sodium channel

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Figure 1. Proximal to distal gradient in FGF14 immunoreactivity in the AIS of multiple CNS neurons

Cryostat sections of the cerebellum were cut from the brains of adult WT ($Fgf14^{+/+}$) mice and mice lacking FGF14 ($Fgf14^{-/-}$) and stained with antibodies against FGF14 and IV-spectrin as described in Materials and Methods. **A.** FGF14 immunostaining (red) is prominent at the AIS of $Fgf14^{+/+}$ Purkinje neurons (arrows) and is higher proximal to the soma and lower distally; immunostaining is also seen in cell bodies of Purkinje neurons (dashed line) and in the AIS of basket/granule neurons (arrowheads). In contrast, no anti-FGF staining is evident in $Fgf14^{-/-}$ cerebellar sections. An antibody against IV-spectrin (green) was used to define the AIS (arrows). **B.** Relative anti-FGF14 (red) and anti-IV-

spectrin (green) fluorescence intensities (FI, mean $\pm$ SEM), determined from linescan analyses of individual *Fgf14*^{+/+} neurons (n = 11) along the AIS. C. Linescan of a single Purkinje neuron along the dashed line shown in (A) demonstrating FGF14 immunofluorescence in the Purkinje neuron soma membrane (arrows). D. Immunostaining of CA1 hippocampal pyramidal neurons reveals a proximal to distal gradient of FGF14 (red) along the AIS (arrows). E. Linescan analysis of anti-FGF14 (red) and anti-IV-spectrin (green) labeling of the AIS in a representative CA1 hippocampal pyramidal neuron. F. Immunostaining of neurons in layer III of the motor cortex reveal a proximal to distal gradient of FGF14 (red) along the AIS (arrows). G. Linescan analysis of anti-FGF14 (red) and anti-IV-spectrin (green) labeling of the AIS in a representative neuron in layer III of the motor cortex. H. Isolated Purkinje neurons maintained in culture for 14 days were stained for FGF14 (red) and IV-spectrin (green), as well as for calbindin (blue; to identify Purkinje neurons). I. High magnification view of an isolated Purkinje neuron showing FGF14 (red) and IV-spectrin (green) immunoreactivity and a proximal to distal gradient similar to that seen in Purkinje neurons *in situ* (A). FGF14 immunostaining can also be seen on the soma (arrows). ML, molecular layer, PL, Purkinje neuron layer; GL, granule neuron layer; PC, Purkinje neuron cell body; scale bars are 10 μ m.

Figure 2. Localization of AIS-associated proteins during Purkinje neuron maturation *in vivo*
 A. Representative images showing immunostaining for FGF14 (red) and IV-spectrin (green) in *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS at postnatal days 1/2, 8/9, and 14/15. B. Representative images of Nav1.6 (red) and ankyrin G (green) immunostaining in *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS at postnatal days 2/3, 8/9 and 14/15. C. Representative images of immunostaining for Nav1.1 (red) and ankyrin G (green) in *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS at postnatal days 2/3, 8/9 and 14/15. The white arrows in each panel indicate the positions of the proximal AIS and the white arrowheads, the distal AIS. In C, the blue arrows mark the distal extent of detectable Nav1.1 immunostaining at the AIS. Scale bars are 10 μm.

Figure 3. Changes in the AIS in *Scn8a^{med}* Purkinje neurons

A. Immunostaining showing FGF14 (green) and IV-spectrin (red) localization in WT and *Scn8a^{med}* (Med) Purkinje neuron AIS. B, C. Line scan analysis showing increased FGF14 immunostaining (B) and similar IV-spectrin immunostaining (C) in *Scn8a^{med}*, compared to WT, Purkinje neuron AIS. Note that the length of the AIS, as demarcated by IV-spectrin immunostaining, is increased in *Scn8a^{med}* Purkinje neurons. D. Immunostaining showing Nav1.1 (red) and ankyrin G (green) localization in WT and *Scn8a^{med}* (Med) Purkinje neuron AIS. E, F. Linescan analysis showing increased Nav1.1 immunostaining (E) and similar ankyrin G immunostaining (F) in *Scn8a^{med}*, compared to WT, Purkinje neuron AIS. Asterisks mark the locations of the Purkinje neuron soma. FI, Fluorescence intensity; ML,

molecular layer, PC, Purkinje neuron soma; PL, Purkinje neuron layer; GL, granule neuron layer. Scale bars are 10 μm . For linescan analyses, n=7–11 values presented are means $\pm$ SEM.

Figure 4. FGF14 and Nav subunit protein levels in cerebellum lacking Nav1.6

Whole cerebellar lysates from WT (+/+) and *Scn8a^{med}* mice were fractionated and Western blots were probed with anti-FGF14, anti-panNav, anti-Nav1.1, and anti-Nav1.6 antibodies. As expected, Nav1.6 protein expression is deficient in *Med* mice cerebellum; However, FGF14, total Nav, and Nav1.1 protein levels are not affected.

Figure 5. Structural components of the AIS are localized normally in Purkinje neurons in adult *Fgf14*^{-/-} mice

A. Immunostaining showing β IV-spectrin (red) and ankyrin G (green) localization in *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS. B. Linescan analysis of ankyrin G in the AIS showing similar intensities. C. Linescan analysis revealed reduced β IV-spectrin labeling in the AIS of *Fgf14*^{-/-}, compared with *Fgf14*^{+/+}, Purkinje neurons. FI, Fluorescence intensity; ML, molecular layer, PC, Purkinje neuron soma; GL, granule neuron layer. Scale bars are 10 μ m. For linescan analysis, n=7–11, error bars equal $\pm$ SEM.

Figure 6. Altered distributions of Nav1.1 and Nav1.6 in *Fgf14*^{-/-} Purkinje neuron AIS

A. Immunostaining showing Nav1.1 (green) and Nav1.6 (red) in the AIS of adult *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neurons. In *Fgf14*^{-/-} Purkinje neurons, the intensity of anti-Nav1.1 immunostaining is decreased and expanded distally (green bracket), compared with the pattern evident in *Fgf14*^{+/+} Purkinje neurons. Similarly, the intensity of anti-Nav1.6 immunostaining is decreased (red bracket) in *Fgf14*^{-/-}, compared with *Fgf14*^{+/+}, Purkinje neurons. Right panels: linescans of the AIS of individual *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neurons. B. Immunostaining showing Nav1.6 (red) and ankyrin G (green) distribution in adult *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS. C. Linescan analysis showing reduced Nav1.6 immunostaining in the AIS of *Fgf14*^{-/-}, compared to *Fgf14*^{+/+}, Purkinje neurons (n = 10 for *Fgf14*^{+/+}, n = 10 for *Fgf14*^{-/-}). D. Immunostaining showing Nav1.1 (green) and ankyrin G (red) distribution in adult *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS. The top graph shows Nav1.1 (FI) vs AIS (μm). The bottom graph shows Nav1.1/AnkG ratio vs AIS (μm). Asterisks (*) indicate significant differences.

= 6 [2], values presented are means $\pm$ SEM). Lower panel: ratio of Nav1.6 to ankyrin G immunofluorescence intensity throughout the AIS. Bracket indicates the region between 6.7 and 10.5 μ m that are significantly ($p < 0.05$) different between *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neurons. D. Immunostaining showing Nav1.1 (red) and ankyrin G (green) localization in adult *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neuron AIS. E. Linescan analysis showing a proximal peak of Nav1.1 immunostaining in *Fgf14*^{+/+} compared to reduced immunostaining in *Fgf14*^{-/-} Purkinje neuron AIS (n=6–10, values presented are means $\pm$ SEM). Lower panel: ratio of Nav1.1 to ankyrin G throughout the AIS. Bracket indicates the region between 0 and 3.9 μ m that are significantly ($p < 0.05$) different between *Fgf14*^{+/+} and *Fgf14*^{-/-} Purkinje neurons. Regions in dashed box are enlarged below. Asterisk marks the location of the Purkinje neuron soma. FI, Fluorescence intensity; ML, molecular layer; PC, Purkinje neuron soma; PL, Purkinje neuron layer; GL, granule neuron layer. Scale bars equal 10 μ m.

Figure 7. Nav1.1 and 1.6 protein levels in whole cerebellar lysates are not affected by loss of FGF14

Cerebellar lysates from four *Fgf14*^{+/+} and four *Fgf14*^{-/-} adult mice were probed with the anti-FGF14, anti-panNav, anti-Nav1.1, anti-Nav1.6 and anti-β-tubulin antibodies.

Figure 8. Protein distribution patterns in the Purkinje neuron AIS in mice lacking FGF14 or Nav1.6

FGF14 (blue) is distributed in a decreasing proximal (P) to distal (D) gradient along the AIS of *Fgf14*^{+/+} cerebellar Purkinje neurons and weakly on the soma membrane. Nav1.1 (green) is distributed in a similar pattern, with high levels proximally and lower levels distally, whereas Nav1.6 (red) is distributed in an inverse pattern with lower levels proximally and higher levels distally. In the absence of FGF14 (*Fgf14*^{-/-}), the intensity of both the anti-Nav1.1 and the anti-Nav1.6 immunolabeling is decreased. In addition, the distributions of these subunits in the AIS is also shifted such that the Nav1.1 domain is expanded and the Nav1.6 domain is constricted. In *Scn8a*^{med} mice, which lack Nav1.6, immunostaining for FGF14 and Nav1.1 at the AIS are increased in parallel with an expanded domain for IV-spectrin, suggesting lengthening of the AIS in *Scn8a*^{med} Purkinje neurons.

Table 1

AIS parameters of wild type (WT) and *Scn8a^{med}* Purkinje neurons

	Length (μm)							
	Ankyrin G	± SEM	IV-spectrin	± SEM	Nav1.1	± SEM	FGF14	± SEM
WT	18.6	0.9	17.8	0.7	3.3	0.4	17.3	1.0
<i>Scn8a^{med}</i>	20.9	0.9	21.7	0.4	8.9	0.3	22.4	0.9
<i>Scn8a^{med}/WT</i>	1.12		1.22		2.70		1.30	
P value	ns		<0.001		<0.01		<0.01	
number (WT, <i>Scn8a^{med}</i>)	(7.7)		(8, 7)		(8, 11)		(7, 8)	

	Fluorescence intensity/μm							
	Ankyrin G ^a	± SEM	IV-spectrin ^a	± SEM	Nav1.1 ^a	± SEM	FGF14 ^a	± SEM
WT	3066	671	6863	959	641	123	7956	1231
<i>Scn8a^{med}</i>	1372	115	5945	737	1690	225	14812	722
<i>Scn8a^{med}/WT</i>	0.45		0.87		2.63		1.86	
P value	<0.05		ns		<0.01		<0.001	
number (WT, <i>Scn8a^{med}</i>)	(7.7)		(8, 7)		(8, 11)		(7, 8)	

^aBackground corrected fluorescence intensity divided by the length of the wild type AIS.

Table 2

AIS parameters in *Fgf14*^{+/+};(WT) and *Fgf14*^{-/-} Purkinje neurons.

	Length (μm)					
	Ankyrin G	± SEM	IV-spectrin	± SEM	Nav1.1	± SEM
<i>Fgf14</i> ^{+/+}	19.4	0.9	19.1	1.0	2.5	0.5
<i>Fgf14</i> ^{-/-}	19.0	0.8	18.8	1.0	6.4	1.4
<i>Fgf14</i> ^{-/-} / <i>Fgf14</i> ^{+/+}	0.98		0.98		2.56	0.93
P value	ns		ns		<0.05	ns
number (WT, <i>Fgf14</i> ^{-/-})	(8, 7)		(8, 7)		(6, 10)	(6, 7)

	Fluorescence intensity/μm							
	Ankyrin Gsup ^a	± SEM	IV-spectrin ^a	± SEM	Nav1.1 ^b	± SEM	Nav1.6 ^b	± SEM
<i>Fgf14</i> ^{+/+}	6358	529	7202	715	6682	919	1019	123
<i>Fgf14</i> ^{-/-}	5237	630	4851	423	2565	375	655	39
<i>Fgf14</i> ^{-/-} / <i>Fgf14</i> ^{+/+}	0.82		0.67		0.38		0.64	
P value	ns		<0.05		<0.001		<0.05	
number (WT, <i>Fgf14</i> ^{-/-})	(8, 7)		(8, 7)		(6, 10)		(6, 7)	

^aBackground corrected fluorescence intensity divided by the length of the *Fgf14*^{-/-} AIS.

^bBackground corrected fluorescence intensity divided by the length of the *Fgf14*^{+/+} (WT) AIS.