Cellular/Molecular

Regulation of $Na_V1.2$ Channels by Brain-Derived Neurotrophic Factor, TrkB, and Associated Fyn Kinase

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Voltage-gated sodium channels are responsible for action potential initiation and propagation in neurons, and modulation of their function has an important impact on neuronal excitability. Sodium channels are regulated by a Src-family tyrosine kinase pathway, and this modulation can be reversed by specifically bound receptor phosphoprotein tyrosine phosphatase-β. However, the specific tyrosine kinase and signaling pathway are unknown. We found that the sodium channels in rat brain interact with Fyn, one of four Src-family tyrosine kinases expressed in the brain. $Na_V1.2$ channels and Fyn are localized together in the axons of cultured hippocampal neurons, the mossy fibers of the hippocampus, and cell bodies, dendrites, and axons of neurons in many other brain areas, and they coimmunoprecipitate with Fyn from cotransfected tsA-201 cells. Coexpression of Fyn with $Na_V1.2$ channels decreases sodium currents by increasing the rate of inactivation and causing a negative shift in the voltage dependence of inactivation. Reconstitution of a signaling pathway from brain-derived neurotrophic factor (BDNF) to sodium channels via the tyrosine receptor kinase B (TrkB)/p75 neurotrophin receptor and Fyn kinase in transfected cells resulted in an increased rate of inactivation of sodium channels and a negative shift in the voltage dependence of inactivation after treatment with BDNF. These results indicate that Fyn kinase is associated with sodium channels in brain neurons and can modulate $Na_V1.2$ channels by tyrosine phosphorylation after activation of TrkB/p75 signaling by BDNF.

Key words: **sodium channel; Fyn; tyrosine kinase; tyrosine phosphorylation; neuromodulation; neurotrophin**

Introduction

Voltage-gated sodium channels generate action potentials in neurons and other excitable cells. Sodium channels are composed of a large pore-forming α subunit (260 kDa) and one or two auxiliary β subunits (33–36 kDa) (Catterall, 2000; Morgan et al., 2000; Yu et al., 2003). Nine genes encode voltage-gated sodium channel α subunits in mammals ($Na_V1.1$ to $Na_V1.9$), and four are expressed primarily in the CNS ($Na_V1.1$, $Na_V1.2$, $Na_V1.3$, and $Na_V1.6$) (Goldin et al., 2000). The $\beta1$ and $\beta3$ subunits are noncovalently associated with the α subunit, whereas the $\beta2$ and $\beta4$ subunits are covalently linked to α by disulfide bonds. The pore-forming α subunit is sufficient for functional expression, but the β subunits are required for the normal kinetics and voltage dependence of channel gating.

Neurotransmitters such as acetylcholine, dopamine, and serotonin reduce sodium channel activity through G-protein-coupled receptors, activation of serine/threonine phosphorylation by PKA and PKC, and subsequent dephosphorylation by calcineurin and phosphoprotein phosphatase 2A (Cantrell and Catterall, 2001). Phosphorylation by PKA and PKC at multiple sites reduces peak sodium currents without a major change in the voltage dependence of activation or fast inactivation (Cantrell and Catterall, 2001). This reduction is caused by enhancement of the intrinsic slow inactivation process of sodium channels (Carr et al., 2003; Chen et al., 2006). Such regulation of sodium channels causes marked changes in the firing properties and input–output relationships of brain neurons (Cantrell and Catterall, 2001; Carr et al., 2003).

Sodium channels are also regulated by protein tyrosine kinases. Acute application of nerve growth factor to PC12 cells, which express $Na_V1.2$ and $Na_V1.7$ channels (D'Arcangelo et al., 1993), reduces sodium current as a result of a negative shift in the voltage dependence of fast inactivation (Hilborn et al., 1998). Activation of exogenously expressed growth factor receptor protein tyrosine kinases, such as tyrosine receptor kinase A (TrkA), basic fibroblast growth factor receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor, also reduces sodium currents in PC12 cells (Hilborn et al., 1998). Receptor phosphoprotein tyrosine phosphatase β (RPTPβ) is specifically bound to sodium channels in rat brain membranes and in transfected cells, and it causes a positive shift in the voltage dependence of sodium channel inactivation and slows the rate of inactivation (Ratcliffe et al., 2000). These results suggest that a signaling complex of sodium channels with tyrosine kinases and phosphatases mediates bidirectional regulation of their activity in response to growth factors and other extracellular signals, but the specific tyrosine kinases and signaling pathways involved in this regulation are unclear. Here, we show that the Src-family tyrosine kinase Fyn associates specifically with sodium channels in rat brain and colocalizes with $Na_V1.2$ channels in numerous loca-

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tions, including the mossy fibers in the hippocampus. Bound Fyn modulates $Na_V1.2$ channels by accelerating fast inactivation and negatively shifting the voltage dependence of fast inactivation, which decreases sodium currents. Reconstitution of signaling from brain-derived neurotrophic factor (BDNF) and TrkB to the $Na_V1.2$ channel via Fyn kinase defines a molecular pathway for sodium channel modulation by neurotrophin receptors through an associated nonreceptor tyrosine kinase in brain neurons.

Materials and Methods

Rat brain membrane preparation. Rats were killed, and their brains were immediately soaked in PBS. Brains were homogenized in 0.32 M sucrose with 5 mM pervanadate and centrifuged at 3000 rpm for 10 min. Supernatants were collected and carefully layered on the top of an equal volume of 1.2 M sucrose and centrifuged at 25,000 rpm for 30 min. The interface layers in the middle of samples were collected and resuspended in 0.8 M sucrose, and the samples were centrifuged at 26,000 rpm for 35 min. Pellets were collected and resuspended in a resuspension buffer containing 25 mM Tris•HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 5 mM pervanadate, and 1% Triton X-100 (TX-100). Aliquots of supernatants were immediately frozen at −80°C. All of the solutions contained a mixture of protease inhibitors [(in μg/ml) 0.685 pepstatin A, 16.56 benzamide, 10 leupeptin, 35 PMSF, and 1 aprotinin] and were kept on ice. The protein concentration of the brain membrane preparations was measured by the BCA assay (Pierce Biotechnology, Rockford, IL).

Expression in mammalian cells. TsA-201 cells were grown in DMEM/F-12 medium (Invitrogen, Rockville, MD), supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin and streptomycin, and incubated at 37°C in 10% CO_2. The cells were grown to ~70% confluence, suspended with trypsin-EDTA, and plated onto 150 mm culture dishes at 40% confluence 24 h before transfection. Before transfection, the medium was replaced with fresh medium. The cells were transiently transfected with 1.2 μg of cDNA encoding $Na_V1.2$ α subunit without or with 100 ng of Fyn cDNA, except where different concentrations are indicated in figure legends, using the calcium phosphate method (Chen and Okayama, 1988; Qu et al., 1994). $Na_V\beta1$ subunits, TrkB, p75, and RPTPβ were cotransfected where indicated in figure legends. cDNA encoding cell surface antigen CD8 (0.8 μg) was included in the transfection mixture for electrophysiological studies, in which successfully transfected cells were identified by binding of anti-CD8-coated beads (Dynal, Oslo, Norway). Cells were incubated overnight at 37°C in 3% CO_2. After 12–16 h, the medium was replaced, and the cells were allowed to recover for 9–24 h.

Membrane preparation from transfected cells. After recovering from transfection, the cells were treated with pervanadate for 5 min at the room temperature. The cells were harvested and lysed in (in mM) 25 Tris•HCl, 150 NaCl, 1 EDTA, 5 pervanadate, and protease inhibitors. The supernatant was collected after centrifugation at 3000 rpm for 1 min, and pellets of the membrane fraction were collected after centrifugation at 26,000 rpm for 30 min. The collected pellets were resuspended, and the protein concentration was measured by the BCA assay.

Immunoprecipitation and immunoblot analysis. Membrane lysates were precleared with protein-A agarose beads (Sigma-Aldrich, St. Louis, MO) for 1 h, and then 500 μg of lysate protein were incubated with 5–25 μg of the appropriate antibody. After 3 h of incubation, protein-A agarose beads were added and incubated overnight. Beads were washed with a washing buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM sodium phosphate, 1 mM pervanadate, 0.01% NaN_3, and 1% Triton X-100) and eluted. Membrane lysates (50 μg) and immunoprecipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ). Blots were probed with antibodies against the family of Src kinases (SRC2; Santa Cruz Biotechnology, Santa Cruz, CA), Src (H-12; Santa Cruz Biotechnology), Fyn (Millipore, Billerica, MA), Lyn (H-6; Santa Cruz Biotechnology), Yes (F-7; Santa Cruz Biotechnology), or sodium channel (anti-SP20) (Westenbroek et al., 1989, 1992), and detected by chemiluminescence (GE Healthcare).

Immunocytochemistry. Neurons from the CA1–CA3 regions of hippocampus from newborn C57BL/6 mice (Harlan, Indianapolis, IN) were mechanically dissociated after 20 min incubation with papain (18.7 U/ml; Worthington Biochemical, Lakewood, NJ). To isolate single cells, the suspension was passed through a 70 μm cell strainer. Neurons were plated at ~20,000 cells/ml on 18 mm coverslips coated with poly-L-lysine and in Neurobasal A media (Invitrogen) supplemented with B27 (1:50), penicillin, and streptomycin (25 U/ml; Invitrogen). Cultures were incubated at 37°C with 5% CO_2 until they were processed for immunocytochemistry.

Transfected tsA-201 cells and cultured hippocampal neurons were washed briefly with 0.1 M sodium phosphate (PB), pH 7.4, and fixed in 4% paraformaldehyde for 45 min. Samples were rinsed in 0.1 M Tris buffer (TB), pH 7.4, for 15 min and in 0.1 M Tris-buffered saline (TBS), pH 7.4, for 15 min and then were blocked in 2% avidin in TBS, rinsed, and incubated in 2% biotin in TBS. Samples were rinsed in TBS for 15 min and then 1% normal serum in TBS containing 0.1% TX-100 for 1 h. Primary antibodies [16 μg/ml anti-α subunit antibody anti-SP20 (Westenbroek et al., 1989;), 15 μg/ml anti-brain type II $Na_V1.2$ (Scn2a) (Millipore), 13 μg/ml anti-Fyn3G (Santa Cruz Biotechnology), 10 μg/ml anti-caveolin-1 (Millipore), and/or 10 μg/ml anti-neurofilament 200 kDa (clone N52; Millipore)] were added, and the coverslips were incubated overnight at 4°C. Samples were rinsed in TBS for 30 min and then incubated with a 1:300 dilution of biotinylated anti-rabbit IgG and a 1:500 dilution of anti-goat IgG conjugated to Alexa-555 and/or 1:500 dilution of anti-mouse IgG conjugated to Alexa-633 for 2 h at room temperature followed by a 2 h incubation with fluorescein-conjugated avidin (1:300), anti-goat IgG conjugated to Alexa-555 (1:500), and/or anti-mouse IgG conjugated to Alexa-633 (1:500) (Vector Laboratories, Burlingame, CA). Coverslips were rinsed in TBS for 10 min, rinsed in TB for 20 min, air-dried, and then mounted on UP-Rite microscope slides (Richard-Allan Scientific, Kalamazoo, MI). They were coverslipped with Vectashield (Vector Laboratories), sealed with nail polish, and visualized under a confocal laser-scanning microscope (SL Confocal; Leica, Bannockburn, IL) located in the W. M. Keck Imaging Facility at the University of Washington.

For staining of hippocampal brain slices, adult rats were anesthetized with halothane and intracardially perfused with a solution of 4% paraformaldehyde in PB. The brain was removed and immersed successively in solutions of 10 and 30% (w/v) sucrose in PB at 4°C over 72 h. The brain was frozen with dry ice, and 40 μm sections were cut on a sliding microtome and soaked in PB buffer. Hippocampal slices were selected, rinsed, and blocked as described above. The sections were then incubated in anti-type II $Na_V1.2$ (Scn2a) antibody (18 μg/ml) and anti-Fyn3G (26 μg/ml) in 1% normal horse serum in TBS containing 0.1% TX-100 for 48 h at 4°C. The sections were rinsed with TBS for 30 min and then incubated with a 1:300 dilution of biotinylated anti-goat IgG and a 1:500 dilution of anti-rabbit IgG conjugated to Alexa-555 for 1 h at 37°C, rinsed again, then incubated with a 1:300 dilution of avidin D-fluorescein and 1:500 dilution of anti-rabbit IgG conjugated to Alexa-555. Tissue sections were then prepared for the imaging as described above.

Electrophysiology. Sodium channels were transiently expressed for electrophysiological analysis in tsA-201 cells as described above. Transfected cells were identified with anti-CD8-coated beads (Dynal) (Jurman et al., 1994). Currents were record in the whole-cell configuration using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Recording solutions were identical to those described previously (Ratcliffe et al., 2000) (in mM): intracellular, 189 *N*-methyl-D-glucamine, 1 NaCl, 4 $MgCl_2$, 0.1 BAPTA, 25 Tris-phosphocreatine, 2 Na-ATP, 0.2 Na-GTP, and 40 HEPES, adjusted to pH 7.2 with H_2SO_4 in the recording pipette; and extracellular, 140 NaCl, 5.4 CsCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, adjusted to pH 7.4 with NaOH in the bath. Recording pipettes were pulled from borosilicate glass transfer tubes and fire polished to resistances of 2–4 MΩ on the day of recording. Data were acquired using Pulse software (Heka, Lambrecht, Germany) controlling an ITC 18 DA/AD interface (Instrutech, Great Neck, NY). All analyses were performed using IGOR Pro (Wavemetrics, Lake Oswego, OR). Standard P/4 subtraction of linear leak and capacitance was used. Series resistance was typically 6–8 MΩ, of which >80% was compensated electronically.

The voltage dependence of activation was measured from a holding

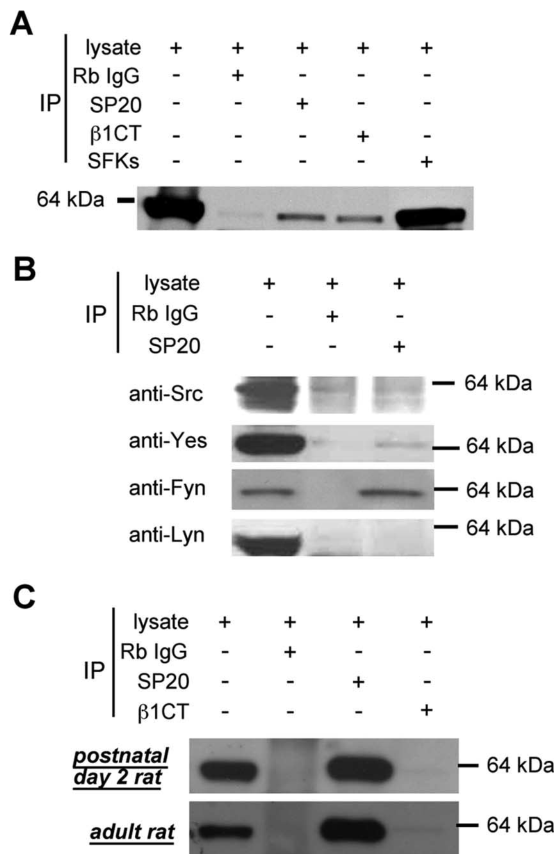

Figure 1. Interaction of $Na_V1.2$ channels with Fyn kinase. ***A***, Rat brain membrane preparations were isolated by sucrose density centrifugation as described in Materials and Methods. Sodium channels were solubilized, and antibodies against the sodium channel α subunit (anti-SP20), the $\beta1$ subunit (anti-$\beta1$CT), or nonimmune rabbit IgG (Rb IgG) were used to immunoprecipitate sodium channel complexes. Antibody against Src-family kinases (SFKs; anti-SRC2) was used to immunoblot the immunoprecipitated (IP) complex. ***B***, Sodium channels from rat brain membranes were solubilized and immunoprecipitated with antibodies against the sodium channel α subunit (anti-SP20) or Rb IgG. The IP complex was immunoblotted with antibodies against individual Src-family kinases (Src, Yes, Fyn, and Lyn, respectively). Each IP sample was from the same membrane lysate to ensure that the same level of each kinase was present in each sample. ***C***, Membrane preparations were isolated from adult rat and postnatal day 2 rat brain. The α and $\beta1$ subunits were immunoprecipitated with anti-SP20 or anti-$\beta1$CT, respectively, and the IP complex was immunoblotted with anti-Fyn antibody. Each experiment was replicated at least three times with consistent results, using the same protocol illustrated in ***A–C***.

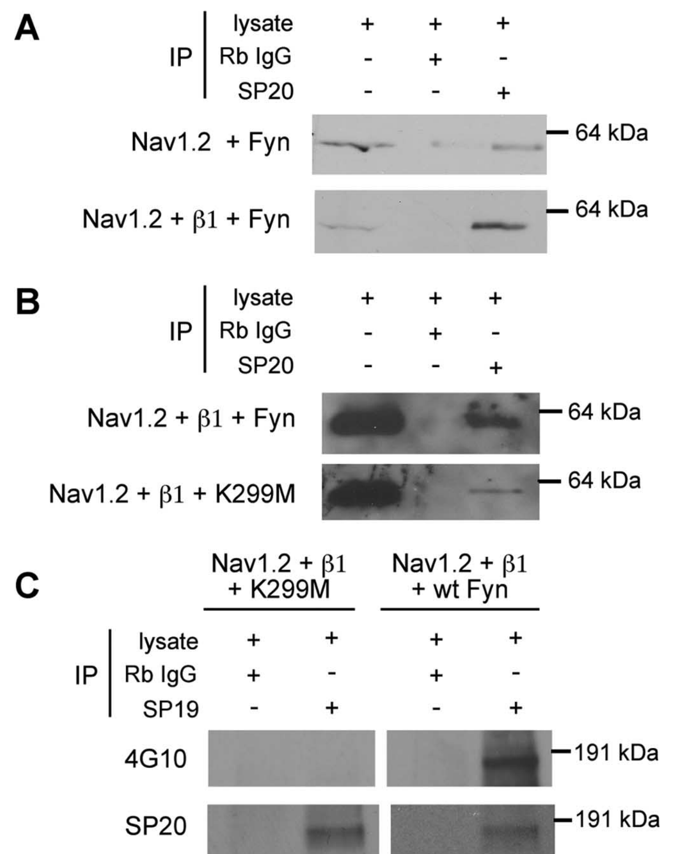

Figure 2. Role of Fyn catalytic activity in association and phosphorylation of $Na_V1.2$ channels. ***A***, $Na_V1.2$ α subunit was expressed in absence or presence of the $\beta1$ subunit cotransfected in a 1:1 molar ratio of cDNA in tsA-201 cells. Membrane lysates were isolated, and anti-SP20 or nonimmune rabbit IgG (Rb IgG) was used to immunoprecipitate sodium channel complexes. The immunoprecipitated (IP) samples were immunoblotted with anti-Fyn. ***B***, $Na_V1.2$ α subunit, $\beta1$ subunit, and either wild-type or catalytically inactive K299M Fyn were coexpressed in tsA-201 cells, and sodium channels were immunoprecipitated with anti-SP20 or nonimmune Rb IgG. Wild-type Fyn (top) and K299M Fyn (bottom) were detected by immunoblotting with anti-Fyn antibodies. ***C***, $Na_V1.2$ α and $\beta1$ subunits were coexpressed with either wild-type (wt) Fyn or K299M Fyn in tsA-201 cells, membranes were isolated, and sodium channel complexes were immunoprecipitated with anti-SP19 or nonimmune Rb IgG. The resulting samples were immunoblotted with anti-phosphotyrosine antibody, 4G10, and then the blot was stripped and reblotted with anti-SP20. Each experiment was replicated at least three times with consistent results, using the same protocol illustrated in ***A–C***.

potential of −110 mV. Cells were depolarized to potentials from −110 to +20 mV in 10 mV increments, and peak inward currents were measured. Activation curves were normalized to maximum peak current. The voltage dependence of inactivation was measured from a holding potential of −110 mV. Cells were depolarized for 100 ms to potentials from −110 mV to −15 mV in 5 mV increments followed by test pulses to −10 mV. Peak sodium currents were normalized and plotted versus prepulse potential. To minimize variability among cells, voltage-clamp protocols were applied with similar sequence and timing to all cells included in each type of experiment.

Results

Association of Src-family kinases with voltage-gated sodium channels in rat brain

Previous studies have shown that sodium channels are modulated by tyrosine kinase signaling via a Src-family kinase (Hilborn et al., 1998) and bound RPTPβ (Ratcliffe et al., 2000). These results raised the possibility that sodium channels form a tyrosine kinase signaling complex containing a bound kinase as well as bound RPTPβ. To examine this possibility, rat brain membrane fractions were isolated and used for coimmunoprecipitation experiments to detect associated Src-family kinases. An immunoreactive Src-family kinase was associated with sodium channels immunoprecipitated by antibodies against the sodium channel α subunit (Fig. 1*A*, lane 3) and the $\beta1$ subunit (Fig. 1*A*, lane 4), but not with control IgG (Fig. 1*A*, lane 2). Because the anti-Src-family antibody recognizes many members of the Src family of protein tyrosine kinases, specific antibodies for each of the four Src-family kinases expressed in brain were tested to identify which tyrosine kinase interacts with the sodium channel. Only Fyn and Yes were coimmunoprecipitated with antibodies against the α subunit (Fig. 1*B*). Relative to the level of the two kinases detected in the lysates, Fyn is more specifically associated with sodium channels than Yes (Fig. 1*B*). Fyn was coimmunoprecipitated with antibodies against the sodium channel α subunit in both adult and neonatal rat brain, but association of Fyn with the $\beta1$ subunit was not detected when these antibodies specific for Fyn were used (Fig. 1*C*). These coimmunoprecipitation experi-

ments provide evidence for the association of Fyn with the α subunit of voltage-gated sodium channels in brain membranes.

Association of recombinant Fyn with $Na_V1.2$ channels in transfected tsA-201 cells

To probe the molecular basis and the functional effects of Fyn interaction with the sodium channel, the α subunit of $Na_V1.2$ channels was coexpressed in tsA-201 cells with B-Fyn, the kinase isoform expressed in the brain. Membrane lysates were isolated, and $Na_V1.2$ channels were immunoprecipitated with anti-SP20 directed against the sodium channel α subunit. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membrane, and a monoclonal antibody against Fyn was used to probe the blot. $Na_V1.2$ channels interacted with Fyn kinase in both the absence and presence of coexpressed $\beta 1$ subunit (Fig. 2*A*). To examine the requirement for Fyn activity, a catalytically inactive Fyn mutant containing the mutation K299M in the ATP-binding pocket (Twamley-Stein et al., 1993; Osterhout et al., 1999) was coexpressed and immunoprecipitated with antibodies against α subunit. Coimmunoprecipitation of mutant Fyn was considerably reduced but not eliminated (Fig. 2*B*). We expect that binding of Fyn to $Na_V1.2$ channels and other substrates is dynamically regulated by phosphorylation of tyrosine residues in SH2-binding domains by Fyn and other tyrosine kinases and by dephosphorylation by phosphoprotein phosphatases (Mustelin and Tasken, 2003), including bound RPTPβ (Ratcliffe et al., 2000). Therefore, these results suggest that the catalytic activity of Fyn increases its binding to $Na_V1.2$ channels by phosphorylating tyrosine residues in SH2-binding motifs, which then increase Fyn binding through interaction with its SH2 domain. Evidence for enhancement of Fyn binding by phosphorylation of SH2-binding motifs in $Na_V1.2$ is presented by Beacham et al. (2007) (accompanying study). We used the 4G10 anti-phosphotyrosine antibody to determine whether $Na_V1.2$ channels are phosphorylated by Fyn bound to them. Tyrosine phosphorylation of $Na_V1.2$ channels was prominently detected when wild-type Fyn was coexpressed (Fig. 2*C*, right), but much less phosphorylation was observed with K299M mutant Fyn (Fig. 2*C*, left). These results indicate that Fyn bound to $Na_V1.2$ channels or an associated protein substantially increases tyrosine phosphorylation of the α subunit.

Colocalization of Fyn with $Na_V1.2$ channels

$Na_V1.2$ channels and Fyn kinase were coexpressed in tsA-201 cells, and the cells were fixed and stained with anti-SP20 and anti-Fyn. When both proteins were expressed, a punctate pattern of localization was observed for both $Na_V1.2$ channel and Fyn in confocal optical sections at the top surface of the cell and near the center of the cell (Fig. 3*A*). A punctate pattern of localization was also observed when Fyn was expressed alone (Fig. 3*B*). In contrast, the punctate pattern of expression was not observed when $Na_V1.2$ channel was expressed alone (Fig. 3*B*), suggesting that association with Fyn was responsible for the punctate pattern of localization of $Na_V1.2$ channels. The average number of $Na_v1.2$ puncta per cell was 1.35 ± 0.55 ($n = 23$) in the absence of Fyn, compared with 33.9 ± 5.5 ($n = 14$; $p < 0.05$) in the presence of Fyn (Fig. 3*D*). Fyn might be clustered in caveolae by means of its N-terminal lipid anchor. Consistent with this hypothesis, clusters of Fyn also contain caveolin (Fig. 3*C*). Therefore, clustering of Fyn in caveolae may be responsible for the punctate localization of coexpressed $Na_V1.2$ channels that interact with Fyn. In any case, the induction of clustering of $Na_V1.2$ channels by Fyn in

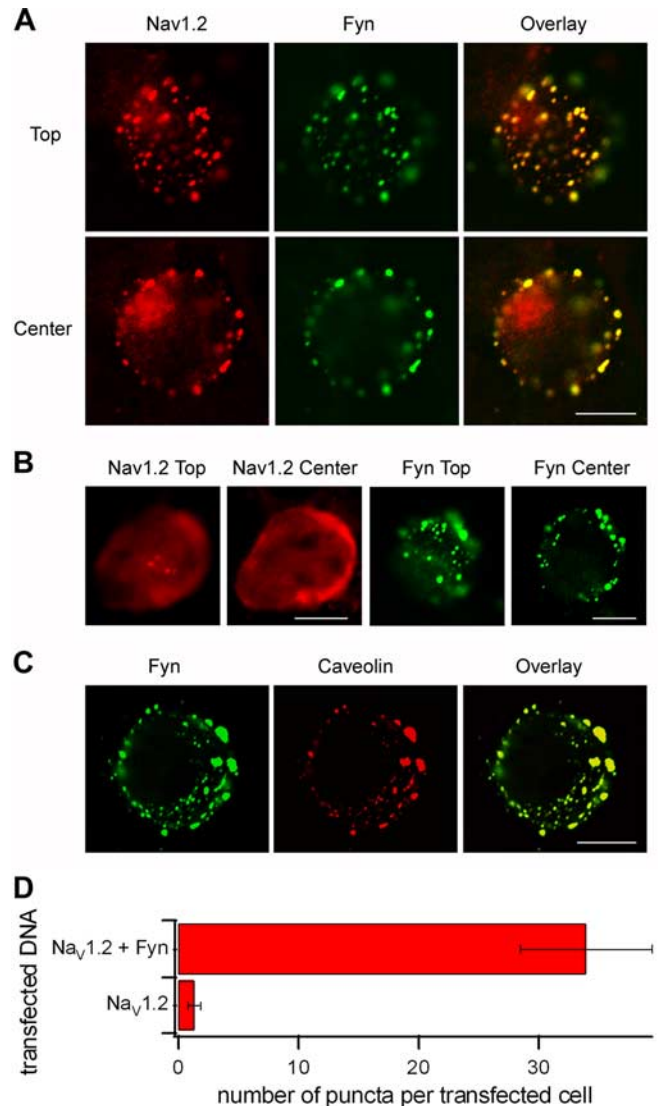

Figure 3. Localization of $Na_V1.2$ and Fyn in transfected tsA-201 cells. ***A***, $Na_V1.2$ and Fyn were coexpressed in tsA-201 cells. The cells were fixed and double immunostained using anti-SP20 and anti-Fyn3G antibodies, as described in Materials and Methods. Optical sections near the top and center of the cell are shown. Regions of overlap are shown in yellow. ***B***, $Na_V1.2$ and Fyn were expressed separately in tsA-201 cells. The cells were fixed and single immunostained using anti-SP20 or anti-Fyn3G antibodies, as described in Materials and Methods. Optical sections near the top and the center of the cell are shown. ***C***, Fyn was expressed alone in tsA-201 cells, and the cells were double immunostained for Fyn and caveolin. Regions of overlap are shown in yellow. Scale bars, 10 μm. ***D***, Cells expressing $Na_V1.2$ only and cells coexpressing $Na_V1.2$ and Fyn were examined in the confocal microscope, and the number of $Na_V1.2$ puncta per cell were counted. The average number of puncta per cell was 1.35 ± 0.55 ($n = 23$) and 33.9 ± 6.5 ($n = 14$) in the absence and presence of Fyn, respectively. Each experiment was replicated at least three times with consistent results, using the same protocol illustrated in ***A–C***. Error bars represent SEM.

cotransfected tsA-201 cells confirms that they interact in intact cells.

Rat hippocampal neurons were cultured and stained with anti-$Na_V1.2$, anti-Fyn, and anti-neurofilament antibodies to examine the localization of these proteins in intact neurons. $Na_V1.2$ channels and Fyn were both expressed along the axons, cell bodies, and dendrites of cultured hippocampal neurons (Fig. 4*A*). Merged images of $Na_V1.2$ channels, Fyn, and the axonal marker neurofilament protein show colocalization of $Na_V1.2$ and Fyn in axons (Fig. 4*B*,*C*). These results indicate that Fyn and $Na_V1.2$

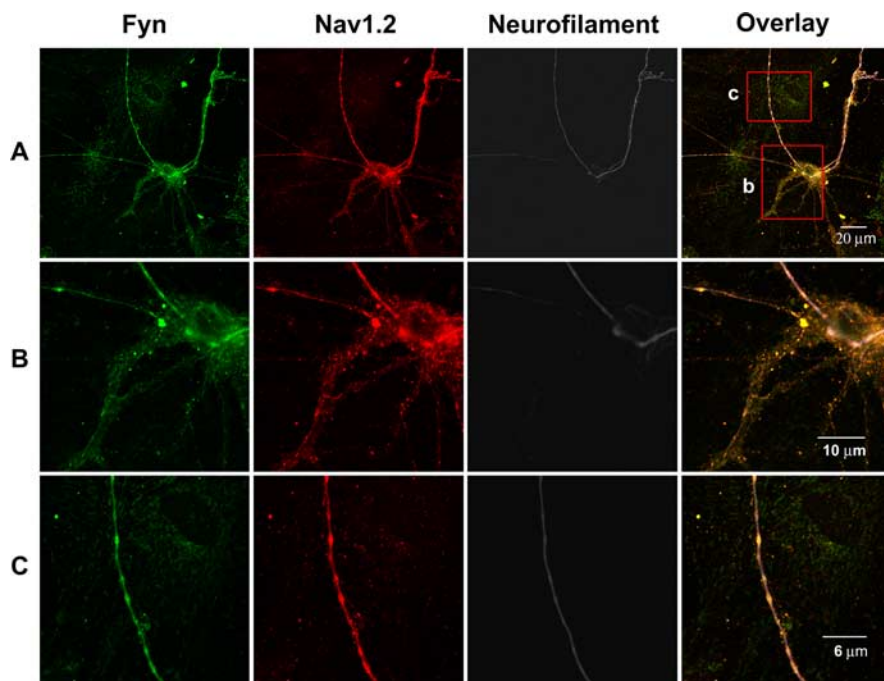

Figure 4. Localization of $Na_V1.2$ and Fyn in cultured rat hippocampal neurons. ***A***, Cultured hippocampal neurons were fixed and triple labeled with anti-Fyn (left, green), anti-$Na_V1.2$ (center left, red), and anti-neurofilament (center right, white). The merged image of $Na_V1.2$ and Fyn is shown on the right. Regions of overlap are shown in yellow. ***B***, ***C***, Magnified images of the boxed areas (b and c, respectively) in ***A*** (right). Scale bars: ***A***, 20 μm; ***B***, 10 μm; ***C***, 6 μm.

channels are localized together in multiple compartments of cultured hippocampal neurons, especially in their axons.

$Na_V1.2$ channels and Fyn are both expressed broadly in brain neurons (Westenbroek et al., 1989; Yagi et al., 1993). To examine whether they are colocalized, we used immunocytochemistry with subtype-specific antibodies. Many areas of colocalization of Fyn and $Na_V1.2$ channels were observed in different brain regions, including the hippocampus, cerebellum, and axonal tracts in brainstem (data not shown). $Na_V1.2$ channels are specifically localized in unmyelinated axons, in contrast to the other brain sodium channel types, $Na_V1.1$, $Na_V1.3$, and $Na_V1.6$ (Westenbroek et al., 1989, 1992; Rasband et al., 2003; Schaller and Caldwell, 2003). To examine colocalization of $Na_V1.2$ channels and Fyn in this specific subcellular location, we focused on the mossy fibers, which project from the dentate granule cells to the CA3 region of the hippocampus. As illustrated in Figure 5*A*, $Na_V1.2$ is localized in the mossy fibers near their termination in the CA3 region of the hippocampus (red, left) and Fyn was also localized in both cell bodies and mossy fibers (green, middle). Many areas of colocalization are observed in the merged image (yellow, right). Higher magnification images illustrate Fyn staining in the mossy fiber tract and mossy fiber terminal region that overlaps with $Na_V1.2$ (Fig. 5*B*,*C*). Interestingly, trkB receptor and its ligand BDNF are also expressed in the mossy fibers and their terminals (Wetmore et al., 1994; Binder et al., 1999; Scharfman et al., 1999). $Na_V1.2$ channels and Fyn are also colocalized in the molecular layer of the cerebellum, where the parallel fibers of granule cells make synapses on the dendrites of Purkinje neurons (Fig. 5*D*). The close colocalization of Fyn and $Na_V1.2$ channels in these axons and nerve terminals may allow local regulation of sodium channel function by neurotrophins acting through Trk receptors and Fyn kinase.

Functional modulation of $Na_V1.2$ channels by Fyn

An important goal of our experiments was to reconstitute the signaling pathway from the neurotrophin receptor to functional modulation of sodium channels in non-neuronal cells. Toward that end, we examined the functional consequences of Fyn regulation of the sodium channel when the α subunit and Fyn were coexpressed in tsA-201 cells, which do not express Fyn (data not shown). In dividing cells like tsA-201 cells, the activity of Fyn is dynamically controlled by tyrosine phosphorylation and dephosphorylation at its multiple regulatory sites (Mustelin and Tasken, 2003). Thus, transfected Fyn kinase is expected to be active, but its activity would be kept below its maximal level by the dominance of phosphoprotein tyrosine phosphatase activity over protein tyrosine kinase activity (Mustelin and Tasken, 2003). Activation of Fyn tyrosine kinase by tyrosine phosphorylation releases its SH2 and SH3 domains to interact with prospective substrates, including the transfected $Na_V1.2$ channel. Therefore, in these experiments, transfected Fyn is expected to interact dynamically with the transfected $Na_V1.2$ channels, phosphorylate them based on the ratio of the activity Fyn kinase to phosphoprotein tyrosine kinases in these cells, and regulate their functional properties. Figure 6*A* (left) shows a family of voltage-activated sodium currents recorded from a transfected cell under whole-cell voltage clamp. At each test pulse potential, sodium currents activate and inactivate within several milliseconds. Coexpression of Fyn accelerates fast inactivation, such that the duration of the sodium current is shorter at each test potential. The time constant for fast inactivation is reduced from 7.3 $\pm$ 0.3 ms ($n = 22$) to 4.3 $\pm$ 0.2 ms ($n = 29$) at -40 mV, and a similar effect is observed at all test pulse potentials negative to -15 mV (Fig. 6*A*, right). Sodium currents are first clearly detected in test pulses to -40 mV and increase to peak values at -10 mV in both the absence and presence of Fyn (Fig. 6*A*, left, *B*). However, the peak sodium current is reduced in cells coexpressing Fyn (Fig. 6*B*) from a mean of 2.8 $\pm$ 0.21 nA at -10 mV in control to 1.8 $\pm$ 0.19 nA at -10 mV. In contrast to the change in peak sodium current, normalized conductance voltage curves (Fig. 6*C*) show that there is no change in the voltage dependence of activation in the presence of Fyn.

The reduction of peak sodium current by Fyn without change in the voltage dependence of activation suggested that the voltage dependence of either fast or slow inactivation of $Na_V1.2$ channels might be negatively shifted by Fyn, thereby contributing to the reduction in peak sodium current. After prepulses of 100 ms duration to membrane potentials more positive than -80 mV, peak sodium currents conducted by $Na_V1.2$ channels are progressively reduced by fast inactivation (Fig. 6*D*, circles). Coexpression of Fyn negatively shifts the voltage dependence of fast inactivation (Fig. 6*D*, squares) from a half-inactivation voltage, V_h, of -64.2 ± 0.4 mV ($n = 40$) in control to -70.1 ± 0.32 mV ($p < 0.01$; $n = 34$) in the presence of Fyn. We found no correlation between the peak current amplitudes in individual cells and

the V_h values, confirming that negatively shifted inactivation is not an artifact resulting from decreased sodium current.

Sodium channels are also regulated on the time scale of seconds by a slow inactivation process, which is distinct from fast inactivation in both time scale and mechanism (Rudy, 1978). Slow inactivation is enhanced by phosphorylation of $Na_V1.2$ channels by PKA and PKC (Carr et al., 2003; Chen et al., 2005). To examine the effects of Fyn kinase on slow inactivation, we applied 1 s depolarizing prepulses to a range of membrane potentials, repolarized to −110 mV for 20 ms to allow complete recovery from fast inactivation, and then measured sodium channel availability for activation in a test pulse to −10 mV. There was no significant difference between control cells and cells coexpressing Fyn kinase in the kinetics or voltage dependence of slow inactivation (Fig. 6*E*) (data not shown). Therefore, the effects of Fyn kinase are specific for fast inactivation.

Reconstitution of a neurotrophin signaling pathway dependent on Fyn kinase

Reconstitution of neurotrophin regulation of sodium channels in a non-neural cell is required to identify the signaling components necessary for modulation. We tested the ability of Fyn kinase to mediate signaling from TrkB receptors to sodium channels in our transfected cell system by coexpressing $Na_V1.2$ channels with TrkB, its modulatory coreceptor p75 (Bothwell, 1995), and Fyn kinase (Fig. 7). The mechanism through which TrkB receptors activate Fyn is not well established, but this pathway regulates NMDA receptors in neurons (Chao, 2003) and may proceed through direct interaction as Fyn binds to TrkB receptors (Iwasaki et al., 1998). In these experiments, we used a lower level of Fyn cDNA (50 ng) in our transfections to reduce baseline Fyn activity and thereby allow us to measure the effect of activation with BDNF more easily. Because regulation of the catalytic activity of Fyn by tyrosine phosphorylation/dephosphorylation is dynamic, we expected that activation of TrkB/p75 by BDNF would increase Fyn activity relative to endogenous tyrosine phosphatases and thereby increase regulation of $Na_V1.2$ channels. When we coexpressed $Na_V1.2$, TrkB, and p75 with Fyn kinase in tsA-201 cells, sodium currents remained constant throughout our typical recording periods after correction for linear rundown (Fig. 7*B*). Addition of 7.1 nM BDNF caused an abrupt, rapid reduction in sodium current (Fig. 7*A*), which approached an apparent steady state at 90% of control with a time constant of 28 s (Fig. 7*B*). The initial rate of decline of the sodium current immediately after addition of Fyn was 6.1-fold more rapid than rundown (Fig. 7*B*, legend).

To observe the full effect of Fyn regulation, we pretreated cells coexpressing TrkB/p75, RPTPβ, and Fyn with 7.1 nM BDNF for 60 min before whole-cell voltage-clamp recording. BDNF treatment reduced peak sodium currents (Fig. 8*A*, squares) without a change in the voltage dependence of activation (Fig. 8*B*). BDNF treatment also negatively shifted the voltage dependence of inactivation (Fig. 8*C*, squares) compared with controls not treated with BDNF (Fig. 8*A*–*D*, circles). The increase in the rate of inactivation (Fig. 8*D*, squares) is smaller than observed in the experiments of Figure 6, in which cells were transfected without or with Fyn, probably because BDNF activation of the lower level of Fyn in the experiments of Figure 8 is not sufficient to overcome the dephosphorylation of the relevant phosphorylation sites by bound RPTPβ, which was cotransfected in the experiments of Figure 8 but not in those of Figure 6. Together, these functional effects reduce sodium channel availability and shorten the duration of sodium currents conducted by those channels that remain available to open. These regulatory effects of BDNF acting through TrkB/p75 and specifically bound Fyn may control sodium channel function locally in axons, nerve terminals, and other subcellular compartments in which these components are colocalized.

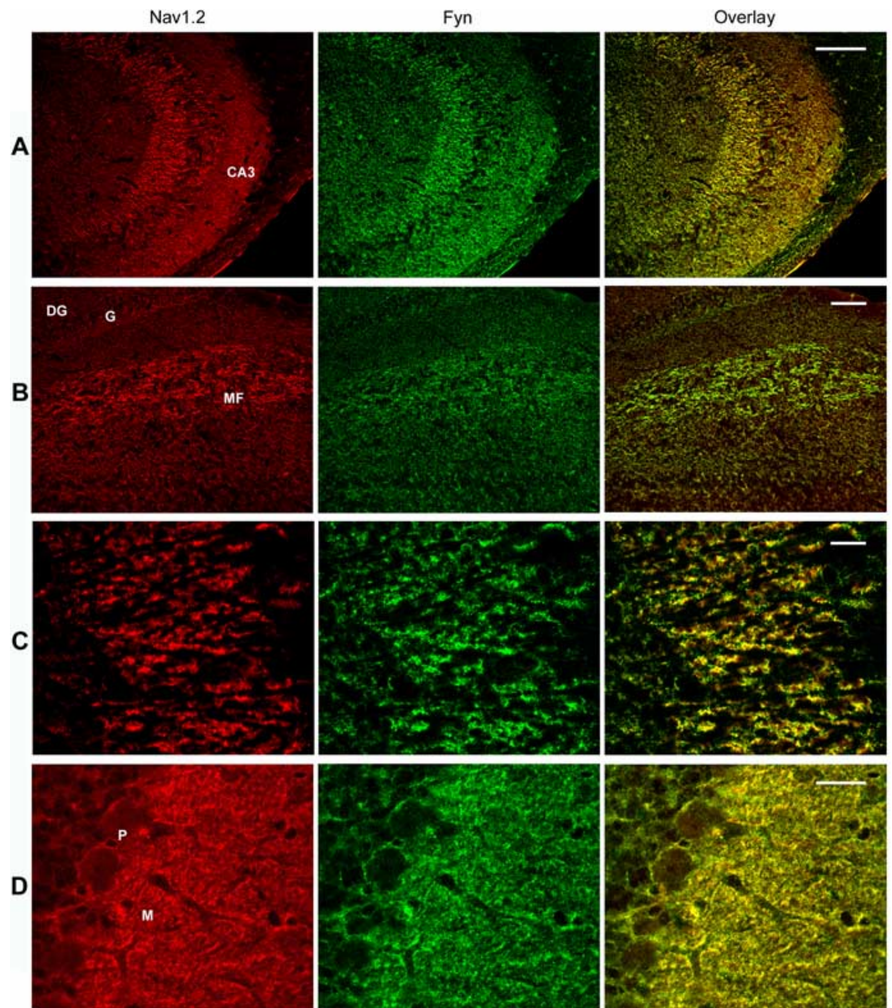

Figure 5. Localization of $Na_V1.2$ channels and Fyn in hippocampus and cerebellum. Sections of rat brain were cut, fixed, and double labeled for $Na_V1.2$ and Fyn as described in Materials and Methods. Shown are $Na_V1.2$ (red, left), Fyn (green, center), and merged images (right). Regions of overlap are shown in yellow. ***A***, CA3 region of the hippocampus. ***B***, Magnified images of the mossy fibers in the dentate gyrus. DG, Dentate gyrus; G, granule cells; MF, mossy fibers. ***C***, High magnification of mossy fibers in the CA3 region. ***D***, Purkinje neurons (P) and molecular layer (M) of the cerebellum. Scale bars: ***A***, 100 μm; ***B***, 50 μm; ***C***, 10 μm; ***D***, 50 μm.

Discussion

Fyn associates with $Na_V1.2$ channels

Our results show that Fyn kinase is associated with sodium channels isolated from rat brain and interacts with $Na_V1.2$ channels

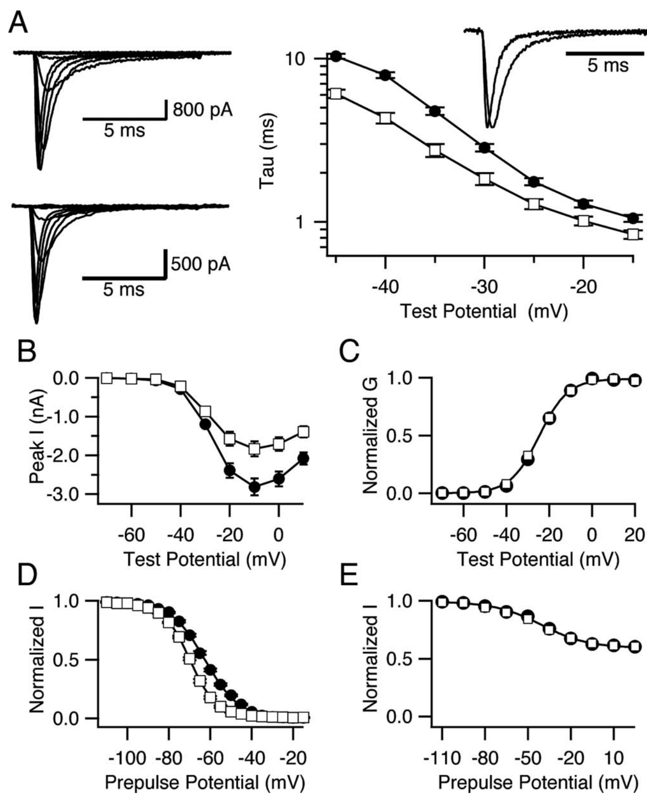

Figure 6. Effects of Fyn expression on functional properties of sodium channels. Sodium currents were recorded from cells transfected with $Na_V1.2$ alone (filled circles) or $Na_V1.2$ plus Fyn (open squares). ***A***, Left, Sodium currents evoked by depolarizations to potentials from −110 to 10 mV in 10 mV steps from a holding potential of −110 mV from cells transfected with $Na_V1.2$ alone (top) or $Na_V1.2$ plus Fyn (bottom). Right, Time course of inactivation during 100-ms-long depolarizations. The decay time course after the peak was fit with a single exponential. Time constants (Tau) are plotted versus test potential. Inset, Normalized sodium currents in response to a depolarization to −15 mV in cells transfected with $Na_V1.2$ alone (slower) and with (faster) Fyn. ***B***, Mean current–voltage relationships. Peak sodium currents were measured during depolarizations to the indicated potentials from cells transfected with $Na_V1.2$ alone (filled circles; $n = 63$) and $Na_V1.2$ plus Fyn (open squares; $n = 46$). ***C***, Mean normalized conductance (*G*)–voltage relationships derived from these current–voltage curves according to $G = I/(V - V_{Rev})$, where *I* is the measured current at voltage *V*, and V_{Rev} is the extrapolated reversal potential. ***D***, Mean normalized inactivation curves. Cells were depolarized for 100 ms with prepulses to the indicated potentials (−110 to −15 mV in 5 mV steps) followed by a 5 ms test pulse to 0 mV. Peak test pulse current was measured and plotted as a function of prepulse potential. Normalized mean peak test pulse current is plotted as a function of prepulse potential. ***E***, Mean normalized slow inactivation curves. Cells were depolarized for 1 s with prepulses to the indicated potentials followed by repolarization for 20 ms to −110 mV to allow recovery from fast inactivation and depolarization to −10 mV for a 10 ms test pulse. Peak test pulse current was measured at each prepulse potential and plotted as a function of prepulse potential. Error bars represent SEM.

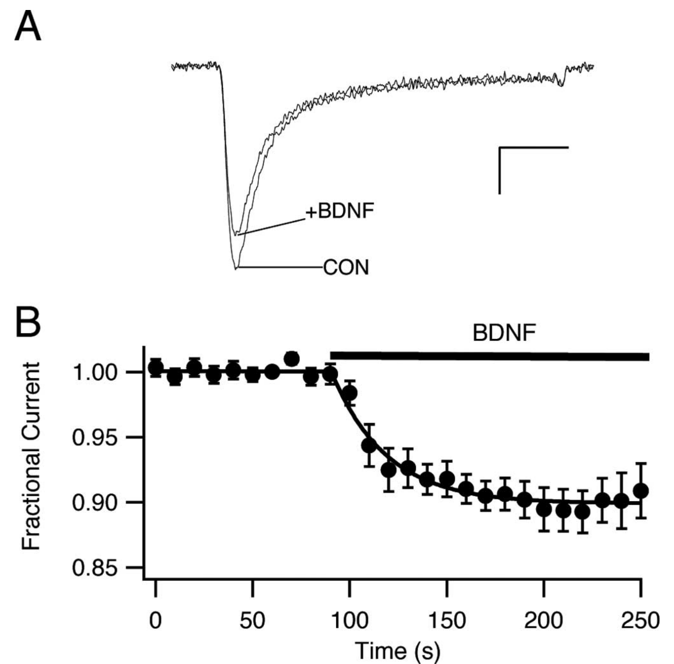

Figure 7. Time course of the effect of BDNF treatment on sodium currents in tsA-201 cells expressing $Na_V1.2$ channels with TrkB, p75, and Fyn. From a holding potential of −70 mV, cells were hyperpolarized to −110 mV for 110 ms, and sodium currents were evoked by 10 ms test pulses to −10 mV before and after addition of 7.1 nM BDNF. ***A***, Example of test pulse sodium currents in control (CON) and after addition of BDNF. Calibration: 500 pA, 2 ms. ***B***, Mean time course of peak sodium current reduction in response to Fyn addition using the same pulse protocol as in ***A***. Linear rundown was subtracted from the individual current records for each cell. The mean linear rundown was 3.4%/min. The BDNF-induced decline in sodium current (***B***, fit curve) had an initial rate of 21.2%/min, 6.1-fold faster than mean rundown. Error bars represent SEM.

when coexpressed in the human embryonic kidney cell line tsA-201. Association with Fyn is specific, in that the closely related Src-family kinases Src and Lck are not associated with sodium channels. In the accompanying study (Beacham et al., 2007), we show that Fyn binds to a specific SH3-binding motif in the intracellular loop connecting domains I and II of the $Na_V1.2$ α subunit and phosphorylates a nearby tyrosine residue as well as tyrosine residues in the inactivation gate, which together are required for regulation of fast inactivation.

The Src-family kinase Yes also is coimmunoprecipitated from rat brain with sodium channels, but a much smaller fraction of endogenous Yes kinase in brain is associated with sodium channels compared with Fyn. Yes may modulate a different subtype of sodium channels or may have different functional effects from Fyn. It will be interesting to compare the modulation of sodium channels by these different Src-family kinases in future work.

Fyn specifically modulates fast inactivation

Voltage-clamp analysis of sodium currents conducted by $Na_V1.2$ channels in the absence and presence of Fyn showed that phosphorylation by Fyn accelerates fast inactivation and shifts its voltage dependence to more negative membrane potentials but has no effect on activation or slow inactivation of sodium channels. These functional effects of Fyn would substantially alter action potential generation in neurons. The negative shift of sodium channel fast inactivation would reduce the availability of sodium channels for activation by depolarizing synaptic currents in neurons and therefore would alter the threshold and frequency of firing, as we have previously shown for enhancement of slow inactivation by phosphorylation of sodium channels by serine/threonine protein kinases (Carr et al., 2003; Chen et al., 2006). The acceleration of inactivation would also reduce excitability by decreasing the inward current drive for depolarization of the cell membrane and by shortening action potentials so that calcium channels are less effectively activated. Together, these two aspects of sodium channel regulation by Fyn would significantly reduce electrical excitability in regions of neurons in which Fyn kinase is bound to sodium channels and is activated by appropriate local signals.

A signaling complex containing $Na_V1.2$ channels

Our previous work showed that $Na_V1.2$ channels are associated with RPTPβ through interactions with both α and β subunits

(Ratcliffe et al., 2000). RPTPβ dephosphorylates the sodium channel α subunit, slows the decay of the sodium current, and shifts the voltage dependence of fast inactivation to more positive membrane potentials (Ratcliffe et al., 2000). These actions are opposite to those of Fyn and likely reflect reversal of tyrosine phosphorylation by Src-family kinases. Thus, our results indicate that sodium channels can be modulated in opposite directions by tyrosine phosphorylation/dephosphorylation catalyzed by bound Fyn and RPTPβ.

The β subunits of sodium channels bind to the extracellular matrix protein tenascin and the cell adhesion molecules contactin and neurofascin (Srinivasan et al., 1998; Xiao et al., 1999; Ratcliffe et al., 2000, 2001; Malhotra et al., 2002; McEwen and Isom, 2004). All of these proteins are involved in cell–cell interactions during axon extension, myelination, and synapse formation. Therefore, $Na_V1.2$ channels in a signaling complex with RPTPβ, tenascin, neurofascin, and contactin may be involved in both electrical signaling and cell–cell interactions during axonal development. $Na_V1.2$ channels are the primary sodium channels in unmyelinated and premyelinated axons (Westenbroek et al., 1989, 1992). In contrast, $Na_V1.6$ channels are the primary sodium channels in nodes of Ranvier of mature myelinated axons (Caldwell et al., 2000; Kaplan et al., 2001; Rios et al., 2003; Suzuki et al., 2004). During the development of myelinated axons, fast inactivation of the sodium current becomes more rapid (Herzog et al., 2003; Rush et al., 2005). Both Fyn regulation of $Na_V1.2$ channels and the switch from $Na_V1.2$ to $Na_V1.6$ channels in the developing nodes of Ranvier may contribute to this developmental effect.

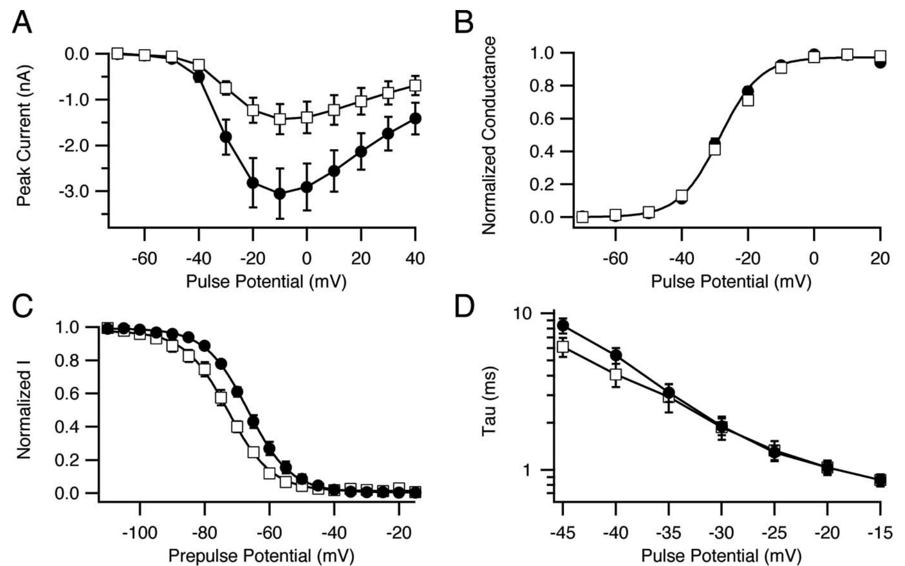

Figure 8. Functional effects of BDNF treatment on cells expressing $Na_V1.2$ channels with TrkB, p75, RPTPβ, and Fyn. ***A***, Mean current–voltage curves from cells expressing $Na_V1.2$, Fyn, TrkB, p75, and RPTPβ in the absence of BDNF (filled circles; $n = 8$) or after exposure to 7.1 nM BDNF for 60 min (open squares; $n = 8$). ***B***, Conductance–voltage relationships, constructed as described in Figure 4*C*, from the data in ***A***. ***C***, Mean normalized inactivation curves as described in Figure 4*D* for cells without (filled circles; $n = 6$) and with BDNF (open squares; $n = 7$). ***D***, Time course of inactivation during depolarizations measured as described in Figure 6*A*. Time constants (Tau) are plotted versus test pulse potential. Error bars represent SEM.

BDNF modulates sodium channels through a neurotrophin signaling pathway involving TrkB, p75, and Fyn

Neurotrophins regulate axon extension, cell–cell interactions, synapse formation, synaptic transmission, and many other aspects of neuronal function (Beggs et al., 1994, 1997; Kimpinski et al., 1999; Osterhout et al., 1999; Yang et al., 2002; Mizuno et al., 2003). Their actions are mediated by the Trk family of receptor tyrosine kinases, often in association with the coreceptor p75 (Bilderback et al., 2001; Chang et al., 2004; Woo et al., 2005), but the signaling pathways that mediate neurotrophin actions and the substrates for neurotrophin regulation are not established in most cases. Our experiments reconstitute a specific neurotrophin signaling pathway from BDNF binding to TrkB/p75, activation of Fyn kinase, phosphorylation of sodium channels, and regulation of sodium channel fast inactivation. This pathway is directly implicated in sodium channel regulation by BDNF and other growth factors in PC12 cells, a sympathetic neuron model (Hilborn et al., 1998). The localization of $Na_V1.2$ channels, BDNF, TrkB, Fyn, RPTPβ, tenascin, neurofascin, and contactin in developing axons and nodes of Ranvier suggests an important role for this signaling pathway in controlling excitability of developing axons.

Control of neuronal excitability by Fyn

Fyn-deficient mice showed enhanced susceptibility to audiogenic seizures, possibly caused by myelination defects (Miyakawa et al., 1995), and Fyn transgenic mice expressing a constitutively activated form of Fyn showed higher seizure susceptibility, possibly caused by changes in regulation of NMDA receptors (Kojima et al., 1997). Expression of BDNF and TrkB receptor is upregulated in mossy fibers in seizure-induced mice (Binder et al., 1999; Scharfman et al., 1999). Disruption of the inhibitory regulation of $Na_V1.2$ channels by Trk receptors and Fyn in these mutant mice may contribute directly to their seizure phenotype. Fine-tuned regulation of sodium channels through Fyn may be essential for control of cellular excitability in addition to its potential role in axonal development and function. The regulation of sodium channels by Fyn may be part of a more extensive regulatory network in which Fyn and other Src-family kinases control neuronal excitability.

Potassium channels, calcium channels, and glutamate receptors are also regulated by Fyn and other Src-family tyrosine kinases. The delayed rectifier potassium channel $K_V2.1$ associates with Fyn and is inhibited by tyrosine phosphorylation (Sobko et al., 1998; Tsai et al., 1999). $K_V1.5$ is tyrosine phosphorylated and interacts with the SH3 domain of Src kinase (Holmes et al., 1996; Nitabach et al., 2001). $K_V1.2$ currents are inhibited by tyrosine phosphorylation (Wischmeyer et al., 1998; Peretz et al., 2000). Fyn also binds and modulates voltage-gated calcium channels (Hou et al., 2003). Therefore, modulation of voltage-gated sodium, calcium, and potassium channels by tyrosine phosphorylation/dephosphorylation may play an important role in mediating cellular plasticity, a form of plasticity that modifies the input–output relationships of the entire neuron (Stuart et al., 1997; Baranauskas and Nistri, 1998; Hausser et al., 2000; Cantrell and Catterall, 2001; Carr et al., 2003). Fyn has been shown to bind and modulate NMDA receptors and synaptic function (Köhr and Seeburg,

1996; Chao, 2003; Yaka et al., 2003), and BDNF increases the excitability of synaptic circuits in the hippocampus and enhances long-term potentiation (Grant et al., 1992; Kojima et al., 1997; Chao, 2003). Modulation of both ion channels and synaptic ionotropic receptors by Fyn may contribute to neurotrophin regulation of cellular excitability.

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