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ORIGINAL PAPER

Src family tyrosine kinase inhibitors suppress Nav1.1 expression in cultured rat spiral ganglion neurons

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Abstract Src family kinases regulate neuronal voltage-gated Na^+ channels, which generate action potentials. The mechanisms of action, however, remain poorly understood. The aim of the present study was to further elucidate the effects of Src family kinases on Nav1.1 mRNA and protein expression in spiral ganglion neurons. Immunofluorescence staining techniques detected Nav1.1 expression in the spiral ganglion neurons. Additionally, quantitative PCR and western blot techniques were used to analyze Nav1.1 mRNA and protein expression, respectively, in spiral ganglion neurons following exposure to Src family kinase inhibitors PP2 (1 and 10 μM) and SU6656 (0.1 and 1 μM) for different lengths of time (6 and 24 h). In the spiral ganglion neurons, Nav1.1 protein expression was detected in the somas and axons. The Src family kinase inhibitors PP2 and SU6665 significantly decreased Nav1.1 mRNA and protein expression ($p < 0.05$), respectively, in the spiral ganglion neurons, and changes in expression were not dependent on time or dose ($p > 0.05$).

Keywords Spiral ganglion neurons · Nav1.1 · Src family kinases · Quantitative PCR · Western blot

Abbreviations

FBS	Fetal bovine serum
PBS	Phosphate buffered saline
PP2	4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*] pyrimidine
PP3	4-Amino-7-phenyl pyrazolo[3,4-*d*] pyrimidine
SFKs	Src family kinases
SGNs	Spiral ganglion neurons
SU6656	2,3-Dihydro-*N*,*N*-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl) methylene]-1H-indole-5-sulfonamide
VGSCs	Voltage-gated sodium channels

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Introduction

Src family kinases (SFKs), members of the non-receptor protein tyrosine kinases, comprise nine members: Src, Fyn, Yes, Lck, Hck, Fgr, Lyn, Yrk, and Blk. Among them, Src, Fyn, and Yes are widely expressed, whereas the remaining SFK show more tissue-restricted expression. For instance, myeloid cells co-express Hck, Fgr, and Lyn, T cells predominantly express Lck and Fyn, and B cells mainly express Lyn, Blk, Fyn, and Fgr (Lowell and Soriano 1996; Abram and Courtneidge 2000). SFKs are involved in cell growth, differentiation, metabolism, signal transduction, and neuronal ion channel and receptor regulation (Cao et al. 2007).

Voltage-gated sodium channels (VGSCs) are mainly distributed in excitable cells and include nine subtypes of alpha subunits from Nav1.1–Nav1.9 (Catterall et al. 2003) that play a critical role in the generation and propagation of action potentials. A large amount of data suggest that voltage-gated Na^+ channels are regulated by SFKs (Ahern et al. 2005; Tong and Stockand 2005; Ahn et al. 2007; Beacham et al. 2007; Jia et al. 2008), and several reports indicate that SFK inhibitors modulate VGSCs. For example, the SFK inhibitor PP2 acts on epithelial Na^+ channels by inhibiting whole cell current and single channel gating. Moreover, results have suggested that SFKs lead to a

negative regulatory cascade resulting in Na^+ channel inhibition (Gilmore et al. 2001). Specifically, Src is essential for growth factor-induced Na^+ channel inhibition, although the influence of Src on Na^+ channels is not direct and occurs via downstream effectors. The activation of Src is a critical component of Na^+ channel regulation in developing and mature nervous systems (Hilborn et al. 1998). Additionally, acute inhibition of SFK by PP2 suppresses Na^+ currents, and a long-lasting block of SFK significantly alters electrophysiological properties of Na^+ channels and reduces the amplitude of Na^+ currents (Francis et al. 2015). However, little information is available concerning the role of SFK in expressional modulation from the α-subunits (Nav1.1–Nav1.9) of VGSCs.

Cochlear spiral ganglion neurons (SGNs), which express Nav1.1, Nav1.6, and Nav1.7, are the primary auditory afferent neurons that encode sound signals into a neuronal code during the primary process of hearing (Fryatt et al. 2009). Earlier studies have shown that SFK inhibition reduces voltage-gated Na^+ current amplitude and delays recovery of Na^+ channels, suggesting that SGN firing behavior, such as discharge frequency and patterns in response to sound stimuli, is critically regulated by SFKs (Feng et al. 2012).

Previous studies on the relationship between SFK and voltage-gated Na^+ channels have primarily focused on electrophysiological properties, with little consideration of Na^+ channel expression. Therefore, in this study we aimed to confirm Nav1.1 mRNA and protein expression and to analyze how SFK inhibitors influence these expression patterns in cultured rat SGNs.

Materials and method

Primary culture of SGN

SGN culture was conducted as described previously (Tang et al. 2010). Briefly, cochlear spiral ganglion tissues were dissected from 3-day-postnatal Sprague–Dawley rats (Experimental Animal Center of Guangxi Medical University). Following cervical decapitation, the cochlea was quickly removed under a stereomicroscope and placed in PBS at 4 °C. The cochlear capsule was then opened. The modiolus containing spiral ganglion was isolated and then minced into small pieces and transferred into the PBS containing 0.25 % trypsin at 37 °C for 10 min. The trypsin was then inactivated by adding DMEM/F12 solution containing 10 % FBS. Following centrifugation at 800(r/min) for 5 min and the removal of supernatant, the pellet was re-suspended and gently triturated. Dissociated tissue was then plated onto poly-D-lysine-coated 35-mm culture dishes and cultured with DMEM/F12 containing 10 % FBS (Gibco, USA) for 48 h in a humidified CO_2 (5 %) incubator (Thermo Forma, USA) at 37 °C. All the animal procedures were consistent with the National Nature Science Foundation of China (NSFC) guidelines.

Immunofluorescence

SGNs cultured on glass coverslips coated with poly-D-lysine were fixed in 4 % paraformaldehyde for 10 min and permeabilized with 0.2 % Triton X-100 for 10 min. After 30 min incubation in bovine serum albumin (10 μg/ml), the cultures were then, respectively, incubated in BSA containing the antibodies (Abcam, UK) against neuronal β-III tubulin (1:300, rabbit) and Nav1.1 (1:1000, mouse) overnight at 4 °C. The double labeled with these antibodies (Cell Signaling Technology, USA) was then visualized with fluorescence Alexa-555 conjugated goat anti mouse (1:300) for Nav1.1, and Alexa-488 conjugated goat anti-rabbit (1:300) for neuronal β-III tubulin, for 1 h at room temperature, respectively. Fluorescence images of SGN were observed under an inverted fluorescence microscope equipped with CellSens Standard software (Olympus, Japan).

Quantitative PCR

Cells were cultured in 35-mm diameter dishes. When PP2 (1 μM, 10 μM), SU6656 (0.1 μM, 1 μM) and PP3 (1 μM, 10 μM) (Sigma, USA) were used, they were added for 6 or 24 h. The methods used for mRNA extraction and cDNA synthesis were similar to that described previously (Tang et al. 2010). In brief, total RNA was extracted using TRIzol reagent (Invitrogen, USA). The mRNA was reverse-transcribed into cDNA with a reagent kit (Takara, Japan). Quantitative PCR was performed using SYBR Green PCR reagents (ABI, USA). The primers for Nav1.1 were 5′-GCTCGGGAGATGTTGTTCC-3′ (forward) and 5′-TCCATCTTGTCATCCTGCAC-3′ (reverse), giving a 106 bp amplicon. The primers for GAPDH were 5′-GACAACTTTGGCATCGTGGA-3′ (forward) and 5′-ATGCAGGGATGATGTTCTGG-3′ (reverse), giving a 133 bp amplicon. The levels of Nav1.1 expression were determined by normalizing to GAPDH expression. The negative control groups contained PCR reagents but without template, which were used to exclude false amplification.

Western blot analysis

The cell lysates were collected, and the protein concentration was determined by the BCA reagents. Equal amounts of cellular proteins (40 μg) boiled together with the loading buffer were fractionated by SDS-PAGE, and transferred to polyvinylidenefluoride (PVDF) membranes (0.45 μm, Millipore, USA) which were blocked in 5 % BSA (diluted in TBST)

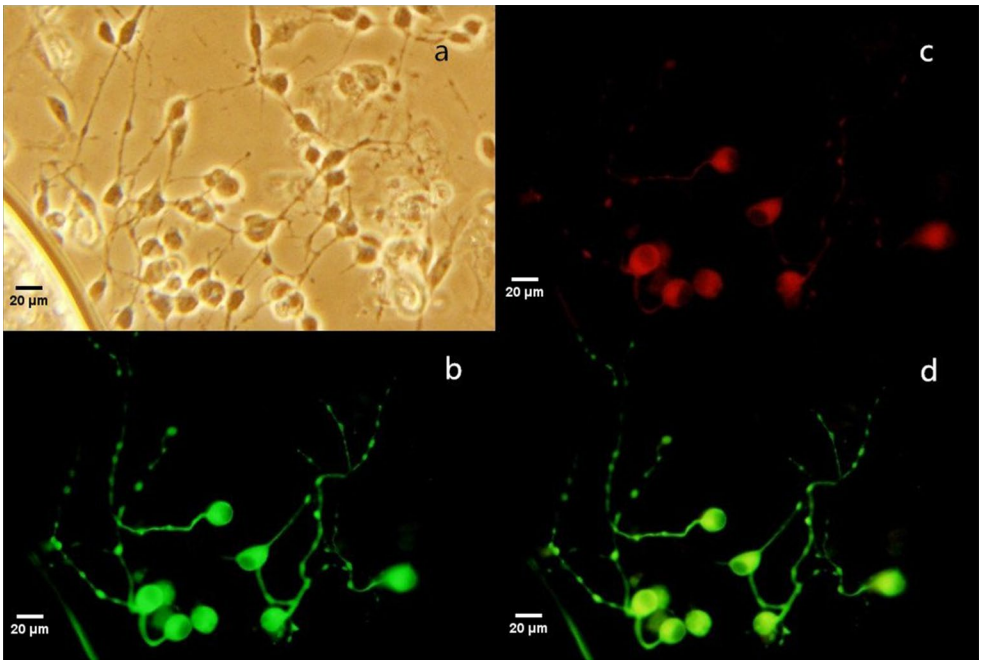


Fig. 1 **a** A view of SGNs under microscope ×400; **b** SGNs were labeled with neuronal class III β-tubulin (*green*), ×400; **c** Nav1.1 were labeled with Nav1.1 antibody (*red*), ×400; **d** merged label neuronal class III β-tubulin and Nav1.1, ×400

for 2 h followed by incubation with different primary antibodies overnight at 4 °C. Antibody against Nav1.1 was used at a concentration of 1:1000, while antibody against GAPDH (KangCheng, China) at 1:10,000. After washed with TBST, the membranes were further incubated with HRP-conjugated secondary antibody (1:10,000, KangCheng, China) for 2 h at room temperature, and finally detected with an electrochemiluminescence system (ECL) (Pierce, USA) by x-ray film. The densitometry of the protein bands was measured using the Gel Image Analysis system (UVP) and normalized to their relevant controls.

Statistics

Data were shown as mean ± SD and analyzed by one-way ANOVA as appropriate to detect statistically significant effects. Statistical differences between means were detected by LSD or SNK test if the ANOVA indicated a significant difference. A p value <0.05 was considered statistically significant.

Results

Nav1.1 location on SGNs

Normal SGNs maintained in culture for 48 h exhibited a large, circular or oval-shaped soma and unipolar or bipolar synapses with smooth cell membranes and a high refraction. The cultures also contained fibroblasts and Schwann cells, among others. Figure 1b–d shows fluorescent microscopy images; SGNs maintained in culture for 48 h were labeled with antibodies specific to neuronal β-III tubulin and Alexa Fluor 488-conjugated secondary antibody (green), as well as antibodies specific to Nav1.1 and Alexa Fluor 555-conjugated secondary antibody (red). Nav1.1 channels and β-III tubulin were expressed in the cell bodies, as well as in the axons of cultured SGNs, although axonal Nav1.1 expression was not as obvious as axonal β-III tubulin expression. Merged images show co-localization of Nav1.1 channels and β-III tubulin protein in cell bodies and axons (Fig. 1).

SFK inhibitors decrease Nav1.1 mRNA expression in SGN

SFK inhibitors PP2 and SU6656 markedly inhibited Nav1.1 mRNA expression compared with control group in all concentrations and treatment times (PP2 group vs. control group: $F = 486.15$, $p = 0.000 < 0.001$; SU6656 group vs. control group: $F = 1801.81$, $p = 0.000 < 0.001$). Exposed to PP2, Nav1.1 mRNA expression in SGN significantly decreased 93.07 % in the 1 μM/6 h group, 89.73 % in the 1 μM/24 h group, 94.54 % in the 10 μM/6 h group and 94.60 % in the 10 μM/24 h group. Meanwhile, after SU6656 treatment Nav1.1 mRNA expression in SGN had

a significant suppression by 92.15 % in the 0.1 μM/6 h group, 91.07 % in the 0.1 μM/24 h group, 92.07 % in the 1 μM/6 h group and 90.05 % in the 1 μM/24 h group. These suppressions were not treatment dose- or time-dependent fashion (PP2 group $p = 0.426 > 0.05$; SU6656 group $p = 0.490 > 0.05$), except that the group of highest PP2 concentration (10 μM) maintained for the longest time (24 h) expressed much less Nav1.1 mRNA than the other experiment groups in our study ($p = 0.005 < 0.05$), indicated that SFK suppressed Nav1.1 mRNA expression in SGN. Application of PP3, an inactive structure analog of PP2, did not induce significant change ($F = 0.174$, $p = 0.967 > 0.05$) in Nav1.1 mRNA expression compared with the control groups (Fig. 2–3).

SFK inhibitors suppress Nav1.1 protein expression in SGNs

Western blot results illustrated of 229 and 36 kD long products, which corresponded to Nav1.1 and GAPDH. Compared with the control group, all concentrations and treatment times of PP2 and SU6656 markedly inhibited Nav1.1 protein expression (PP2 group vs. control group: $F = 16.079$, $p = 0.000 < 0.001$; SU6656 group vs. control group: $F = 96.787$, $p = 0.000 < 0.001$). PP2 treatment resulted in significantly decreased Nav1.1 protein expression in SGNs (30.97 % in the 1 μM/6 h group, 28.13 % in the 1 μM/24 h group, 27.34 % in the 10 μM/6 h group, and 45.88 % in the 10 μM/24 h group). SU66565 treatment also resulted in significantly suppressed Nav1.1 protein expression in SGNs (30.14 % in the 0.1 μM/6 h group, 29.39 % in the 0.1 μM/24 h group, 25.92 % in the 1 μM/6 h group, and 34.68 % in the 1 μM/24 h group). These reductions were not treatment dose- or time-dependent (PP2 group, $p = 0.73 > 0.05$; SU6656 group, $p = 0.08 > 0.05$). However, the group with the highest PP2 concentration (10 μM) that was maintained for the longest time (24 h) expressed much less Nav1.1 protein than the other experiment groups in our study ($p = 0.021 < 0.05$), suggesting that SFK suppressed Nav1.1 protein expression in SGNs. Conversely, PP3 did not induce significant change ($F = 0.95$, $p = 0.484 > 0.05$) in Nav1.1 protein expression compared with the control group (Fig. 4).

Discussion

Na^+ channels influence cellular electrophysiological activity

Na^+ channels, a type of transmembrane glycoproteins, are involved in the generation and transmission of action potentials and are classified into three types according to their method of activation: voltage-gated sodium channels, concentration-gated sodium channels, and acid-sensitive ion channels(Waldmann and Lazdunski 1998). VGSCs are sub-divided into nine categories according to the various α-subunits (Nav1.1–Nav1.9) (Catterall et al. 2003).

Previous data showed that Nav1.1 is expressed in different cells and influences cellular electrophysiological activity. For instance, Nav1.1 influences signal transmission among neurons in the optic chiasm and is able to alter circadian rhythm in mice (Han et al. 2012). Mice with a BACE1 [β-site APP-cleaving enzyme 1 (APP: amyloid precursor protein)] deletion exhibit decreased brain Nav1.1 expression and induced seizures (Kim et al. 2011a, b). Additionally, a Nav1.1 gene mutation has been shown to play a role in Dravet syndrome (severe myoclonic epilepsy in infancy) (Volkers et al. 2011).

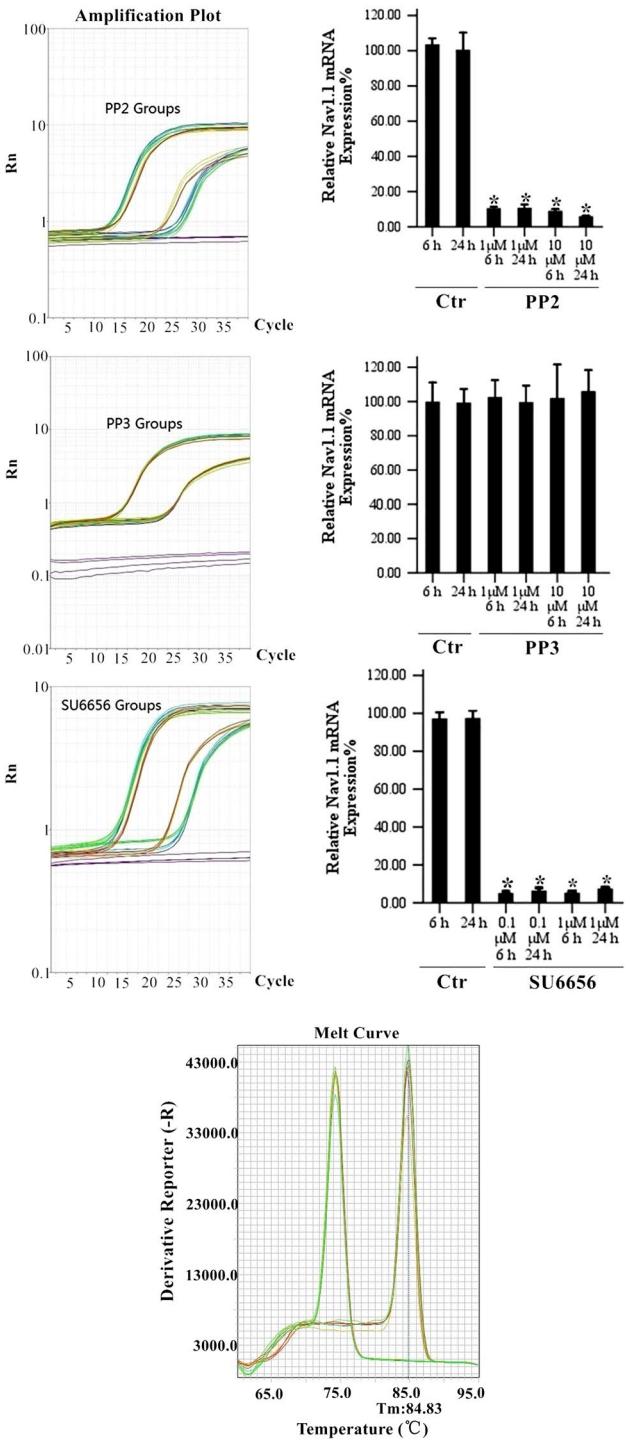
Previous studies have reported Nav1.1, Nav1.6, and Nav1.7 expression in adult rat SGNs (Fryatt et al. 2009), which are the auditory primary and predominately afferent neurons that serve as the hub between hair cells and the central auditory system. In a noise-induced hearing loss rat model, Nav1.1 expression in SGNs was decreased by 29 % (Fryatt et al. 2011). Using the patch-clamp technique, we were previously able to measure Na^+ current on rat SGNs (Feng et al. 2012). Results from the present study revealed Nav1.1 expression in SGNs. Together, these results indicate that not only is Nav1.1 expressed in rat SGNs, but also it is closely related to the generation and conduction of action potentials.

Cochlea from postnatal rat pup has had adult feature by birth

Although the length of various mammalian gestations varies, the basic rule of cochleas' occurrence and development appeared almost identical. The cochleas of embryonic animals have had their adult structure and feature by birth. For example, development of the rat inner ear begins with thickening of the ectoderm area at E8, followed by an otocyst at E9, otocyst and ossicular chain at E9.5, and the formation of a cochlear duct anlage by E12.5. Corti's formation can be seen at E16, and cochlear tube development is complete by E18.5. By birth, the structure and function of the rat inner ear is fully developed. Therefore, SGN cultures from postnatal rat pups in the present study are assumed to correlate with adult SGN cultures (Kim et al. 2006).

Src family kinases regulate multiple mRNA expression

SFKs, a class of non-receptor protein tyrosine kinases, are involved in signaling pathways in a variety of cells. In the aspect of RNA levels, SFK inhibitor PP1 inhibited MMP-9,



◀**Fig. 2–3** Effects of SFKs inhibitors, PP2, SU6656, and PP3 on Nav1.1 mRNA expression in SGNs. A B C D E F G *Color A* means Control 6 h group. *Color B* means Control 24 h group. *Color C* means Treatment group (1 μM PP2 6 h group/1 μM PP3 6 h group/0.1 μM su6656 6 h group). *Color D means* Treatment group (1 μM PP2 24 h group/1 μM PP3 24 h group/0.1 μM su6656 24 h group). *Color E* means Treatment group (10 μM PP2 6 h group/10 μM PP3 6 h group/1 μM su6656 6 h group). *Color F* means Treatment group (10 μM PP2 24 h group/10 μM PP3 24 h group/1 μM su6656 24 h group). *Color G* means Negative control group, these controls were with PCR reagents, but without sample. *Melt Curve*: Single peak models indicated a good amplification specificity. The melting temperatures (*Tm*) corresponded to Nav1.1 and GAPDH were 74.98 and 84.83 ℃. *Rn* (normalized reporter) is the reporter signal normalized to the fluorescence signal of reference dye. *$p < 0.05$ vs. control group (*Ctr*), ($\bar{x} \pm s$, $n = 3$)

c-Fos and c-Jun mRNA in rat brian astrocytes (Yang et al. 2012). Moreover, PP2 reduced STAT signaling activation, fibronectin and TGF-β1 mRNA expression in cultured human proximal tubular epithelium cells (Hamzeh et al. 2015). PP2 also inhibited COX-2 and PGE_2 mRNA expression in primary gingival keratinocytes(Chang et al. 2014). Afterward, PP1 and SU6656 markedly attenuated heme oxygenase-1 gene expression in human tracheal smooth muscle cells (Yang et al. 2015).

Src family kinases regulate multiple receptors and ion channels

In the field of regulating various receptors and ion channels in different cells, SFK appeared quite a few commonality in the detailed mechanism, such as G-protein-coupled receptors (GPCR) (Gavi et al. 2006; McGarrigle and Huang 2007; Almendro et al. 2010), acetylcholine receptors (AchR) (Kim et al. 2011a, b), NMDA receptors (Salter and Kalia 2004; Groveman et al. 2012), GABA receptors (Brandon et al. 2001; Zhang et al. 2010), K^+ channels (Sobko et al. 1998; Ling et al. 2000, 2004; Strauss et al. 2002; Li et al. 2004), and Na^+ channels (Ahern et al. 2005; Tong and Stockand 2005; Ahn et al. 2007; Beacham et al. 2007; Jia et al. 2008).

Src family kinases and non-ion channel receptors

GPCR internalization promoted by Src agonist has been previously confirmed. Src was used to stimulate the β_2 adrenergic receptor (β_2AR), a member of GPCR, resulting in increased intracellular cAMP, activation of PKA and G-protein-coupled receptor kinases, and β_2AR phosphorylation of Ser/Thr residues. The phosphorylation accelerated the combination of β_2AR with β-arrestin and clathrin, resulting in desensitization and internalization. This study suggested that the β-arrestin–Src complex serves as a major factor in the internalization and desensitization process (Gavi et al. 2006; McGarrigle and Huang 2007; Almendro et al. 2010).

A dynamic balance between SFK-mediated protein tyrosine phosphorylation and phosphotyrosine phosphatase-mediated dephosphorylation is crucial for regulating nAChR activity and, ultimately, synaptic excitability. Additionally, different specific isoforms of nAChRs result in variable outcomes of SFKs action, and these responses

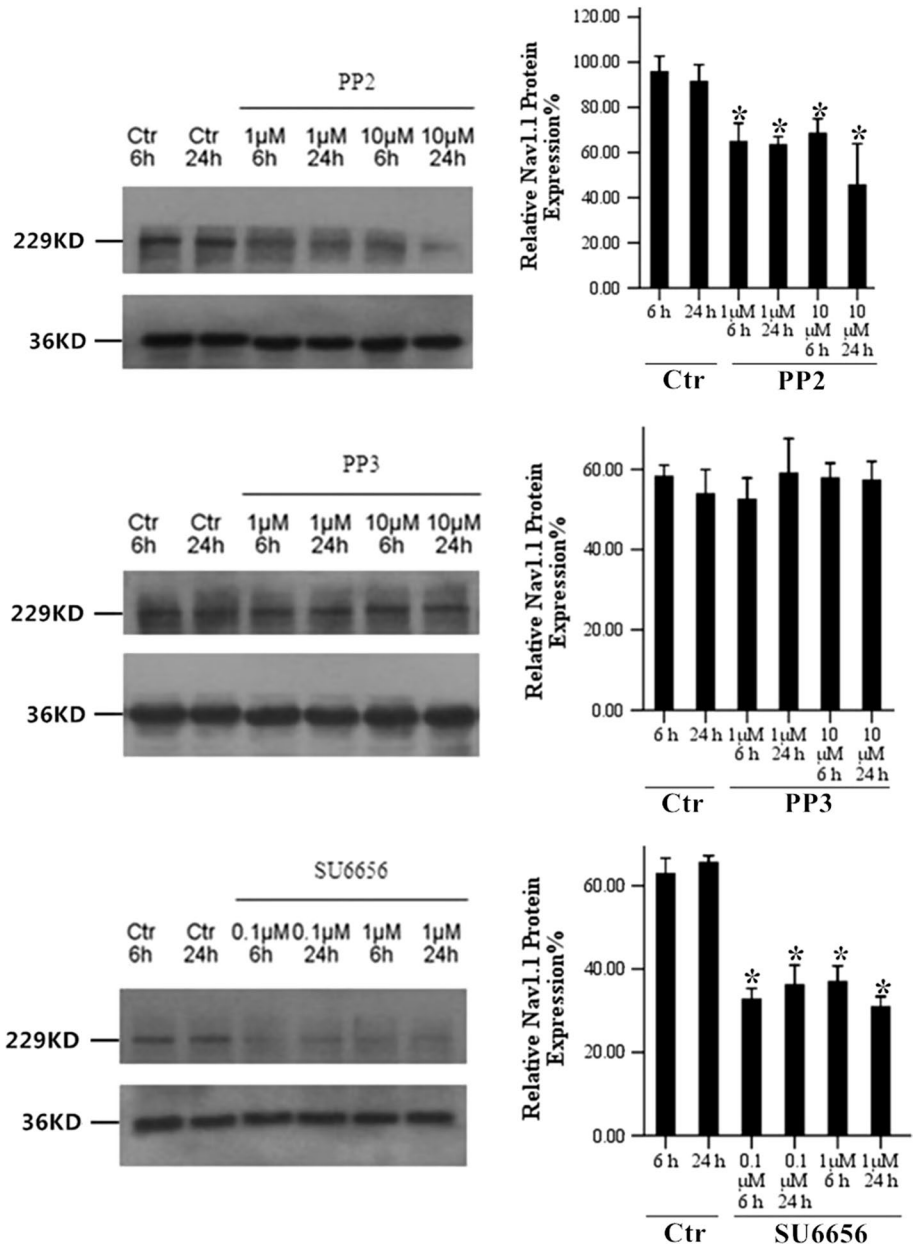


Fig. 4 Effects of SFKs inhibitors, PP2, SU6656, and PP3 on Nav1.1 protein expression in SGNs. $*p < 0.05$ vs control group (*Ctr*), ($\bar{x} \pm s$, $n = 3$)

are dependent on the degree of phosphorylation. For instance, α7 homomeric nAChRs are deactivated by tyrosine phosphorylation in the brain, whereas α3β4 nAChRs are positively regulated by SFKs in pelvic ganglion neurons. Additionally, the activation of α3β4 nAChRs induces fast depolarization followed by consistent hyperpolarization, as well as the shuttling of $[Ca^{2+}]$i through $[Na^{+}]$i-dependent membrane depolarization (Kim et al. 2011a, b).

Src family kinases and ion channel receptors

SFKs play a significant role in multiple, diverse signaling pathways, as well as the regulation of NMDA receptor functions. When NMDA receptors are activated by SFKs through tyrosine phosphorylation, there is a rapid increase in NMDA receptors expression on the cellular surface. Further studies have shown that NMDA receptors form a complex with Src by binding to PSD-95. The SH2 domain of Src is thought to connect with PSD-95 via the PDZ1 domain, and PDZ1 binds to the NR2 subunit of NMDA receptor via the ESDV motif. The PSD-95 protein serves as a negative mediator in Src-regulation of the NMDA receptor. Conversely to the NR2A and NR2B subunits, however, tyrosine phosphorylation was not identified in the NR1 subunit of NMDA receptors (Salter and Kalia 2004; Groveman et al. 2012).

Earlier studies have demonstrated that tyrosine phosphorylation of the β2/3 and γ2 subunits within the GABAA receptor increases when SFK becomes activated, thereby potentiating whole cell and synaptic responses in the CNS. Furthermore, phosphorylation of tyrosine residues 365 and 367 in the γ2 subunit induced by Fyn enhances synaptic inhibition as a result of the negative regulation of GABAA receptor endocytosis (Brandon et al. 2001; Groveman et al. 2012). Conversely, activated SFK has been shown to inhibit GABAB receptor transmitter release in primary afferents. When SFK phosphorylates the Cav2.2 channel unique splice variant of 37a exon, which contains a tyrosine residue susceptible to SFK phosphorylation, this process inhibits Cav2.2 and reduced the amount of Ca^{2+} entering the terminal through Cav channels. This ultimately decreased substance P release in primary afferent neurons and relieves neuropathic pain, such as trigeminal neuralgia (Zhang et al. 2010).

Src family kinases and ion channels

Voltage-gated Na^+ and K^+ channels remain a primary determinant of neuronal excitability and activity. Previous reports have shown that SFK effects on Na^+ and K^+ channels are not always similar; some are enhanced, while some are weakened. This is dependent on channel structure, intracellular signaling, and even cell types. Studies have shown that SFKs influence voltage-gated Na^+ or K^+ currents when different types of channels are expressed, such as on cervical ganglion neurons, renal cells, Schwann cells, vascular smooth muscle cells, tsA-201 cells, and HEK-293 cells. Endogenous SFK can be mediated by a variety of mechanisms, thereby exhibiting different enzyme activities and activation states. Protein phosphorylation plays a role in the determination of ion channel activity by altering expression on the cell surface or channel gating. However, it remains to be determined how Na^+ or K^+ channel types are identified and regulated by each SFK member, as well as whether surface Na^+ or K^+ channel expression or gating control is regulated by SFK (Sobko et al. 1998; Ling et al. 2000, 2004; Strauss et al. 2002; Li et al. 2004; Ahern et al. 2005; Tong and Stockand 2005; Ahn et al. 2007; Beacham et al. 2007; Jia et al. 2008; Feng et al. 2012).

We have previously reported that voltage-gated Na^+ currents in SGNs are consistently down-regulated and delay recovery from inactivation by PP2 and SU6656, but not by PP3 (Feng et al. 2012). However, the mechanisms by which Src inhibitors suppress voltage-gated Na^+ currents in rat SGNs remain poorly understood. Fyn, another non-receptor protein tyrosine kinases, interacts with I and II homologous domains of Nav1.2 via the SH3 domain and further phosphorylates tyrosine residues to modulate Nav1.2 by accelerating inactivation and negatively shifting voltage dependence, ultimately decreasing Na^+ current (Beacham et al. 2007). Additionally, Fyn kinase coordinates binding of Nav1.2 channels and mediates inhibition of Na^+ currents by enhancing fast inactivation in rat hippocampal neurons by phosphorylating domains I and II tyrosine residues of Nav1.2 (Ahn et al. 2007). Tyrosine phosphorylation of Na^+ channels results in hyperpolarization and steady-state inactivation, thereby reducing available channels for action potentials (Hilborn et al. 1998; Ratcliffe et al. 2000).

SFK inhibition of Na^+ currents, however, exhibits only an acute SFK blocking effect on Na^+ channel activation, instead of a lasting SFK suppression on Nav mRNA and protein expression. The present study was performed to determine how SFK is involved in the sustained regulation of Nav mRNA and protein expression in SGNs. Our results showed that PP2 or SU6656 resulted in decreased Nav1.1 mRNA and protein expression in rat SGNs. Following Src inhibition, Nav1.1 mRNA expression was blocked, resulting in decreased protein expression. This was consistent with previous findings showing that SFK inhibited epithelial Na^+ channels and suggested that several mechanisms could be involved where SFK might inhibit Nav1.1 expression. First, SFK activation might increase Nav1.1 phosphorylation and subsequent expression. Second, SFK activation might alter phosphatase activity that subsequently dephosphorylates the channel or an associated regulator. Third, SFK might induce cytoskeletal rearrangements that affect the association of stimulatory or inhibitory proteins with Nav1.1. Finally, the target of SFK activity could be a Nav1.1-associated cytosolic protein, and its binding or release from the channel might potentially alter Nav1.1 expression. Any of these models could account for the lasting effects of Src family kinases on Nav1.1 expression (Gilmore et al. 2001).

It is also possible that SFK-induced Nav1.1 mRNA and protein expression in SGNs is the basis for cellular functional change that controls Na^+ currents and results in altered SGN

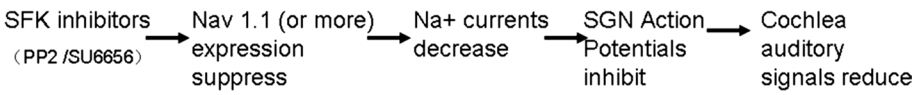


Fig. 5 Proposed model to illustrate that Src inhibitors-reduced auditory signals from cochlea SGNs is mediated through Nav1.1 (or more subunits) expression suppression/Na^+ currents decrease/SGNs action potential inhibition

action potentials. Based on suggestion from our findings and on the literature, Fig. 5 exhibits a model for the molecular mechanisms underlying Src inhibitors induced a decrease of action potentials and auditory signals in cochlea SGNs. This eventually regulates functions of the inner ear, such as sound transmission, differentiation, and tuning.

Future studies will explore whether VGSCs are influenced by the SFKs-induced phosphorylation of tyrosine residues, as well as to determine the sites that SFKs effect on. The present study only analyzed Nav1.1 expression in relation to Na^+ currents, and the role of Nav1.6 and Nav1.7 needs further analysis, as well as the downstream effectors in ion channel regulation, which will provide a better understanding of issues related to VGSCs in the inner ear.

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References

Abram CL, Courtneidge SA (2000) Src family tyrosine kinases and growth factor signaling. Exp Cell Res 254:1–13

Ahern CA, Zhang JF, Wookalis MJ, Horn R (2005) Modulation of the cardiac sodium channel NaV1.5 by Fyn, a Src family tyrosine kinase. Circ Res 96:991–998

Ahn M, Beacham D, Westenbroek RE, Scheuer T, Catterall WA (2007) Regulation of Na(v)1.2 channels by brain-derived neurotrophic factor, TrkB, and associated Fyn kinase. J Neurosci 27:11533–11542

Almendro V, Garcia-Recio S, Gascon P (2010) Tyrosine kinase receptor transactivation associated to G protein-coupled receptors. Curr Drug Targets 11:1169–1180

Beacham D, Ahn M, Catterall WA, Scheuer T (2007) Sites and molecular mechanisms of modulation of Na(v)1.2 channels by Fyn tyrosine kinase. J Neurosci 27:11543–11551

Brandon NJ, Delmas P, Hill J, Smart TG, Moss SJ (2001) Constitutive tyrosine phosphorylation of the GABA(A) receptor gamma 2 subunit in rat brain. Neuropharmacology 41:745–752

Cao Z, George J, Baden DG, Murray TF (2007) Brevetoxin-induced phosphorylation of Pyk2 and Src in murine neocortical neurons involves distinct signaling pathways. Brain Res 1184:17–27

Catterall WA, Goldin AL, Waxman SG, International Union of P (2003) International Union of Pharmacology. XXXIX. Compendium of voltage-gated ion channels: sodium channels. Pharmacol Rev 55:575–578

Chang MC, Chen YJ, Chang HH, Chan CP, Yeh CY, Wang YL, Cheng RH, Hahn LJ, Jeng JH (2014) Areca nut components affect COX-2, cyclin B1/cdc25C and keratin expression, PGE2 production in keratinocyte is related to reactive oxygen species, CYP1A1, Src EGFR and Ras signaling. PLoS One 9:e101959

Feng S, Pflueger M, Lin SX, Groveman BR, Su J, Yu XM (2012) Regulation of voltage-gated sodium current by endogenous Src family kinases in cochlear spiral ganglion neurons in culture. Pflugers Arch 463:571–584

Francis KR, Wei L, Yu SP (2015) SRC tyrosine kinases regulate neuronal differentiation of mouse embryonic stem cells via modulation of voltage-gated sodium channel activity. Neurochem Res 40:674–687

Fryatt AG, Vial C, Mulheran M, Gunthorpe MJ, Grubb BD (2009) Voltage-gated sodium channel expression in rat spiral ganglion neurons. Mol Cell Neurosci 42:399–407

Fryatt AG, Mulheran M, Egerton J, Gunthorpe MJ, Grubb BD (2011) Ototrauma induces sodium channel plasticity in auditory afferent neurons. Mol Cell Neurosci 48:51–61

Gavi S, Shumay E, Wang HY, Malbon CC (2006) G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. Trends Endocrinol Metab 17:48–54

Gilmore ES, Stutts MJ, Milgram SL (2001) SRC family kinases mediate epithelial Na^+ channel inhibition by endothelin. J Biol Chem 276:42610–42617

Groveman BR, Feng S, Fang XQ, Pflueger M, Lin SX, Bienkiewicz EA, Yu X (2012) The regulation of *N*-methyl-D-aspartate receptors by Src kinase. FEBS J 279:20–28

Hamzeh MT, Sridhara R, Alexander LD (2015) Cyclic stretch-induced TGF-beta1 and fibronectin expression is mediated by beta1-integrin through c-Src- and STAT3-dependent pathways in renal epithelial cells. Am J Physiol Renal Physiol 308:F425–436

Han S, Yu FH, Schwartz MD, Linton JD, Bosma MM, Hurley JB, Catterall WA, de la Iglesia HO (2012) Na(V)1.1 channels are critical for intercellular communication in the suprachiasmatic nucleus and for normal circadian rhythms. Proc Natl Acad Sci USA 109:E368–377

Hilborn MD, Vaillancourt RR, Rane SG (1998) Growth factor receptor tyrosine kinases acutely regulate neuronal sodium channels through the src signaling pathway. J Neurosci 18:590–600

Jia Z, Jia Y, Liu B, Zhao Z, Jia Q, Liang H, Zhang H (2008) Genistein inhibits voltage-gated sodium currents in SCG neurons through protein tyrosine kinase-dependent and kinase-independent mechanisms. Pflugers Arch 456:857–866

Kim HJ, Kang KY, Baek JG, Jo HC, Kim H (2006) Expression of TGFbeta family in the developing internal ear of rat embryos. J Korean Med Sci 21:136–142

Kim DY, Gersbacher MT, Inquimbert P, Kovacs DM (2011a) Reduced sodium channel Na(v)1.1 levels in BACE1-null mice. J Biol Chem 286:8106–8116

Kim NH, Park KS, Cha SK, Yoon JH, Yeh BI, Han KH, Kong ID (2011b) Src family kinase potentiates the activity of nicotinic acetylcholine receptor in rat autonomic ganglion innervating urinary bladder. Neurosci Lett 494:190–195

Li Y, Langlais P, Gamper N, Liu F, Shapiro MS (2004) Dual phosphorylations underlie modulation of unitary KCNQ K(+) channels by Src tyrosine kinase. J Biol Chem 279:45399–45407

Ling S, Woronuk G, Sy L, Lev S, Braun AP (2000) Enhanced activity of a large conductance, calcium-sensitive K+ channel in the presence of Src tyrosine kinase. J Biol Chem 275:30683–30689

Ling S, Sheng JZ, Braun AP (2004) The calcium-dependent activity of large-conductance, calcium-activated K+ channels is enhanced by Pyk2- and Hck-induced tyrosine phosphorylation. Am J Physiol Cell Physiol 287:C698–706

Lowell CA, Soriano P (1996) Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev 10:1845–1857

McGarrigle D, Huang XY (2007) GPCRs signaling directly through Src-family kinases. Sci STKE 2007:pe35

Ratcliffe CF, Qu Y, McCormick KA, Tibbs VC, Dixon JE, Scheuer T, Catterall WA (2000) A sodium channel signaling complex: modulation by associated receptor protein tyrosine phosphatase beta. Nat Neurosci 3:437–444

Salter MW, Kalia LV (2004) Src kinases: a hub for NMDA receptor regulation. Nat Rev Neurosci 5:317–328

Sobko A, Peretz A, Attali B (1998) Constitutive activation of delayed-rectifier potassium channels by a Src family tyrosine kinase in Schwann cells. EMBO J 17:4723–4734

Strauss O, Rosenthal R, Dey D, Beninde J, Wollmann G, Thieme H, Wiederholt M (2002) Effects of protein kinase C on delayed rectifier K+ channel regulation by tyrosine kinase in rat retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 43:1645–1654

Tang XL, Gao M, Feng S, Su JP (2010) Gamma-aminobutyric acid A receptor and *N*-methyl-D-aspartate receptor subunit expression in rat spiral ganglion neurons. Neural Regeneration Research 5:1–3

Tong Q, Stockand JD (2005) Receptor tyrosine kinases mediate epithelial Na(+) channel inhibition by epidermal growth factor. Am J Physiol Renal Physiol 288:F150–161

Volkers L, Kahlig KM, Verbeek NE, Das JH, van Kempen MJ, Stroink H, Augustijn P, van Nieuwenhuizen O, Lindhout D, George AL Jr, Koeleman BP, Rook MB (2011) Nav 1.1 dysfunction in genetic epilepsy with febrile seizures-plus or Dravet syndrome. Eur J Neurosci 34:1268–1275

Waldmann R, Lazdunski M (1998) H(+)-gated cation channels: neuronal acid sensors in the NaC/DEG family of ion channels. Curr Opin Neurobiol 8:418–424

Yang CM, Lin CC, Lee IT, Lin YH, Yang CM, Chen WJ, Jou MJ, Hsiao LD (2012) Japanese encephalitis virus induces matrix metalloproteinase-9 expression via a ROS/c-Src/PDGFR/PI3K/Akt/MAPKs-dependent AP-1 pathway in rat brain astrocytes. J Neuroinflammation 9:12

Yang CM, Lin CC, Lee IT, Hsu CK, Tai YC, Hsieh HL, Chi PL, Hsiao LD (2015) c-Src-dependent transactivation of EGFR mediates CORM-2-induced HO-1 expression in human tracheal smooth muscle cells. J Cell Physiol 230:2351–2361

Zhang G, Chen W, Marvizon JC (2010) Src family kinases mediate the inhibition of substance P release in the rat spinal cord by mu-opioid receptors and GABA(B) receptors, but not alpha2 adrenergic receptors. Eur J Neurosci 32:963–973