

Multiple Nav1.5 isoforms are functionally expressed in the brain and present distinct expression patterns compared with cardiac Nav1.5

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Abstract. It has previously been demonstrated that there are various voltage gated sodium channel (Nav) 1.5 splice variants expressed in brain tissue. A total of nine Nav1.5 isoforms have been identified, however, the potential presence of further Nav1.5 variants expressed in brain neurons remains to be elucidated. The present study systematically investigated the expression of various Nav1.5 splice variants and their associated electrophysiological properties in the rat brain tissue, via biochemical analyses and whole-cell patch clamp recording. The results demonstrated that adult Nav1.5 was expressed in the rat, in addition to the neonatal Nav1.5, Nav1.5a and Nav1.5f isoforms. Further studies indicated that the expression level ratio of neonatal Nav1.5 compared with adult Nav1.5 decreased from 1:1 to 1:3 with age development from postnatal (P) day 0 to 90. This differed from the ratios observed in the developing rat hearts, in which the expression level ratio decreased from 1:4 to 1:19 from P0 to 90. The immunohistochemistry results revealed that Nav1.5 immunoreactivity was predominantly observed in neuronal cell bodies and processes, whereas decreased immunoreactivity was detected in the glial components. Electrophysiological analysis of Nav1.5 in the rat brain slices revealed that an Na current was detected in the presence

of 300 nM tetrodotoxin (TTX), however this was inhibited by $\sim 1 \mu\text{M}$ TTX. The TTX-resistant Na current was activated at -40 mV and reached the maximum amplitude at 0 mV . The results of the present study demonstrated that neonatal and adult Nav1.5 were expressed in the rat brain and electrophysiological analysis further confirmed the functional expression of Nav1.5 in brain neurons.

Introduction

Voltage-gated sodium channel (Nav) 1.5 is important in the generation and propagation of action potentials, in working myocardium and cardiac tissue conduction cells (1), therefore, it is considered the primary cardiac Na channel. However, the present study, in accordance with data from previous studies, demonstrated that Nav1.5 was expressed in various mammalian tissues including the brain, neuronal cell lines (2-14), dorsal root ganglia (DRG) (15-17), gastrointestinal tract (18), and various tumor tissues and cell lines (19-32), in addition to its established presence in cardiac tissue. Currently, a total of nine distinct Nav1.5 channel isoforms, Nav1.5a-f and the truncated variants Nav1.5 E28B-D, have been identified. It has been demonstrated that four of these variants, including Nav1.5a and Nav1.5c-e, may act as functional channels generating an Na current (1,33,34). Therefore, it is important to verify the distinct Nav1.5 isoforms expressed in different tissues, in order to completely elucidate the specific functional contribution to each tissue type.

The authors previously demonstrated the expression of neonatal Nav1.5 (Nav1.5e), Nav1.5a and Nav1.5f in the rat brain (9,10,13). However, the presence of further Nav1.5 isoforms expressed in the rat brain and their exact localization remains to be elucidated. The function of Nav isoforms in these tissues also remains to be elucidated. Therefore, the present study systematically investigated the expression of distinct Nav1.5 isoforms in the frontal lobe of Sprague Dawley (SD) rat brains via the reverse transcription-polymerase chain reaction (RT-PCR), DNA sequencing, restriction enzyme

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digestion and immunocytochemistry. Following this, the functional properties of Nav1.5 in the frontal lobe brain slices were analyzed using whole-cell patch clamping. In addition, the expression patterns of various Nav1.5 isoforms in the rat brain were detected and compared with those present in the rat heart.

Materials and methods

Materials. The investigation was approved by the Ethics Committee and the Committee of Animal Experimentation of China Medical University (Shenyang, China) and Capital Medical University (Beijing, China). Healthy male Sprague-Dawley rats at postnatal day (P)0-90 [total n=54; age groups: P0 (n=6); P3 (n=6); P6 (n=6); P9 (n=6); P12 (n=6); P15 (n=6); P21 (n=6); P30 (n=6); P90 (n=6)] were provided by the Animal Experimentation Center of Capital Medical University (Beijing, China). They were housed with 2 of each group/cage and maintained on a 12-h light/dark cycle with food and water available *ad libitum* under a constant temperature (23±2°C). Rats were anesthetized with sodium pentobarbital (30 mg/kg body weight) and were sacrificed by cervical dislocation. Tissues used for RT-PCR and immunocytochemistry were carefully excised. The rat brain used for the patch clamp experiment (P21-30; weight 200-250 g) was dissected and placed in ice-cold, oxygenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF; pH 7.4; 126 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 0.5 mM CaCl₂, 5 mM MgCl₂, 1.25 mM NaH₂PO₄ and 25 mM dextrose).

RNA isolation and RT-PCR. Total RNA was extracted from the cortical layers of the frontal lobe of the SD rat brain tissue at differing developmental stages. The RNeasy lipid tissue Mini kit (Qiagen, Inc., Valencia, CA, USA) was used to extract the total RNA according to the manufacturer's protocol. The cDNAs were synthesized using the SuperScript[®] VILO™ cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For the detection and isolation of different Nav1.5 isoforms from the frontal lobe of the rat brain, the primer pairs 1-3 (Table I) were used to amplify different fragments of Nav1.5 cDNA via PCR, according to the manufacturer's protocol (New England Biolabs, Inc., Ipswich, MA, USA). PCR using Platinum™ Taq DNA polymerase (Thermo Fisher Scientific, Inc.) was performed separately according to the specific re-annealing temperatures of the different primers. The PCR reaction conditions were as follows: 95°C for 5 min followed by 36 cycles of 95°C for 30 sec, 60-66°C for 30 sec and 72°C for 30 sec, then final elongation at 72°C for 5 min. PCR products were analyzed by gel electrophoresis (1-2% agarose). The signal of each band was determined using Quantity One 4.6 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gene expression ratios of Nav1.5 splicing variants were presented as amplicon densities of Nav1.5 to its variants. All PCRs for detecting the relative amount of Nav1.5 and its splicing variants were repeated at least three times.

DNA sequencing. All PCR products were separated by electrophoresis on a 2% agarose gel. The different fragments of expected size (240, 367 and 373 bp) were extracted and purified using a gel extraction kit (Qiagen, Inc.) and then sequenced

directly using a 3730xl DNA sequencer (Applied BioSystems; Thermo Fisher Scientific, Inc.).

Restriction enzyme digestion. In order to distinguish the neonatal splice variant of Nav1.5 from the total Nav1.5 cDNA present, restriction enzyme SacI was used to digest the total PCR products. The reaction system was 30 µl in total, containing 4 µl PCR products, 0.5 µl SacI enzyme, 3 µl loading buffer and 22.5 µl super-purified water. The electrophoresis was performed on 2% agarose gel to detect the digestion results following a 1 h incubation period at 38°C. The expression ratio of Nav1.5 variants vs. total Nav1.5 was detected from the signal quantification of pre- and post-digestion using a gel imaging analysis device (ChemiDoc MP; Bio-Rad Laboratories, Inc.). The signal of each band was determined using Quantity One v4.6 software (Bio-Rad Laboratories, Inc.).

Immunohistochemistry assay. Fresh specimens of rat brain tissue from P0-90 were fixed with 4% paraformaldehyde (4°C for 24 h) immediately following collection. The brain tissues were processed by dehydration using a series of graded ethanol baths (70-100% ethanol), clearing (the transparency of tissue) using xylene (100%) and wax infiltration and were then paraffin-embedded. The streptavidin-peroxidase immunohistochemical method was applied. Immunohistochemistry was performed using the Histostain-SP kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Sections (4-µm) were incubated overnight with rabbit Nav1.5 primary antibody (1:100; cat. no. ASC-013; Alomone, Jerusalem, Israel) at 4°C in a moisture chamber and were then washed three times with PBS for 5 min. Sections were then incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:500; cat. no. #656140; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 30 min and washed three times with PBS for 5 min. PBS replaced the primary antibody to serve as the negative control and the slide with known positive Nav1.5 expression in rat atrial muscle served as the positive control. The immunohistochemical staining results were observed using a light microscope (Olympus CX31-LV320; Olympus Corporation, Tokyo, Japan). The expression of brown and yellow staining on the cells was considered to indicate positive immunoreactions when compared with negative and positive slices.

Western blot analysis. A total of 100 mg tissue was rinsed with pre-cooled PBS (4°C) and mixed with 500 µl radio-immunoprecipitation assay strong lysate buffer (Pierce radioimmunoprecipitation Buffer; cat. no. #89900; Thermo Fisher Scientific, Inc.). A protease inhibitor cocktail (Pierce; Thermo Fisher Scientific, Inc.) was subsequently added at a volume ratio of 1:100 and the mixture was homogenized and lysed at 4°C overnight. Following centrifugation at 16,100 x g for 30 min at 4°C, the supernatants were collected and assayed for protein content. The protein concentration was determined using the bicinchoninic acid method and 100 µg of protein was loaded into each lane for a 6-10% SDS-PAGE running at a constant voltage of 80 V for 2 h. The proteins were subsequently transferred onto a polyvinylidene fluoride membrane at a constant current of 400 mA for 2 h. The membrane was blocked with 10% non-fat milk for 2 h and incubated with

Table I. Primer sequences used for the isolation of Nav1.5 variants in the rat brain.

Primer pair	Sequence (5' to 3')		Targeting	Locationa	Length (bp)
	Forward	Reverse			
1	5'-TTTCTGCTGCATGCATTACACCTT-3'	5'-GCAGAAGACAGTGAGGACCA-3'	Exon6/6A(Nav1.5&Nav1.5e)	724-963	240
2	5'-GTGCCCCCAGCCCCGCAAGGAAA-3'	5'-TGCTGCCCTCGGAGTAACCTGT-3'	Nav1.5a, Nav1.5c	3101-3467	367
3	5'-TTCAGCGCAGACAACCTCACA-3'	5'-TGTTCTCTTCAATCCTCTTCCT-3'	Nav1.5d	2870-3242	373

^aRelative to nucleotide A of the start codon ATG in rat brain Na voltage-gated channel α subunit 5. Nav, voltage gated sodium channel.

rabbit anti-Nav1.5 polyclonal antibody overnight at 4°C (1:200; Alomone). The membrane was rinsed with TBS Tween-20 three times and incubated with horseradish peroxidase-linked anti-rabbit IgG secondary antibody (1:2,000; cat. no. #7074; CST Biological Reagents Company Limited, Shanghai, China) with shaking at room temperature for 1 h, and subsequently exposed and developed using the ECL system. Nav1.5 immunoreactive protein bands were detected with an enhanced chemiluminescence reagent (ECL-Plus) and densitometrically quantitated according to the manufacturer's protocol (GE Healthcare Life Sciences, Chalfont, UK). Experiments were repeated at least three times.

Electrophysiological recordings. The experiments were performed at room temperature. Following anesthetization, the rat brain (P21-30) was removed and placed in ice-cold ACSF, which was continuously bubbled with 95% O₂ and 5% CO₂. Brain slices of the frontal lobe were cut on a vibratome (250-300 μ m; VT 1000 S; Leica Microsystems, Inc., Buffalo Grove, IL, USA) in ice-cold cutting ACSF and separated into the left and right hemispheres. The slices were transferred into a chamber containing ACSF and incubated with a mixture of 95% O₂ and 5% CO₂ for 30-45 mins at 36°C. The slices were kept at room temperature for the subsequent experiment. Whole-cell patch-clamp recordings were performed on layer V pyramidal neurons of the frontal lobe with a HEKA EPC-10 patch-clamp amplifier with associated software (PULSE version 8.3 Software for Data Acquisition; IGOR Pro5.03 for Graphing and Data Analysis; HEKA Elektronik, Inc., Pfalz, Germany). The pipette solution contained 145 mM CsCl, 2 mM MgCl₂, 2 mM Na₂ATP, 10 mM HEPES, 0.2 mM EGTA and 2 mM tetraethylammonium (TEA). To isolate the Na⁺ currents, CdCl₂ (200 μ M) and TEA (20 mM) were added to the bath solution. Differing concentrations of tetrodotoxin (TTX) (10, 100 and 300 nM, and 1 μ M; cat. no. A0224, CAS no. 4368-28-9; Mansite Biotechnology Co., Ltd, Chengdu, China) were added to the bath solution to identify TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) Na currents.

Results

Expression of Nav1.5 mRNA splice variants in the frontal lobe of rat brain

Adult and neonatal Nav1.5 (Nav1.5e) are expressed in the frontal lobe of rat brain and ventricular myocytes of rat heart. Primer pair 1 targeting exon 6 and exon 6A was used to detect the expression of adult Nav1.5 and neonatal Nav1.5 (Nav1.5e) in the frontal lobe of the rat brain at differing developmental ages. The results revealed that a single band with the expected size was observed on 2% agarose gel (Fig. 1A). Direct DNA sequencing of the purified PCR products demonstrated that a single sequence presented in the exon 5 or 7 coding regions; however, a dual sequence appeared in the exon 6/6A coding regions (Fig. 1B). DNA sequence analysis revealed that exon 6 and 6A of Na voltage-gated channel α subunit 5 (SCN5A) were inclusively expressed, indicating that adult (wild-type) and neonatal Nav1.5 (Nav1.5e) variants were expressed in the frontal lobe of the rat brain.

In order to quantify the expression of the two splice variants of Nav1.5, the restriction enzyme SacI was used to digest

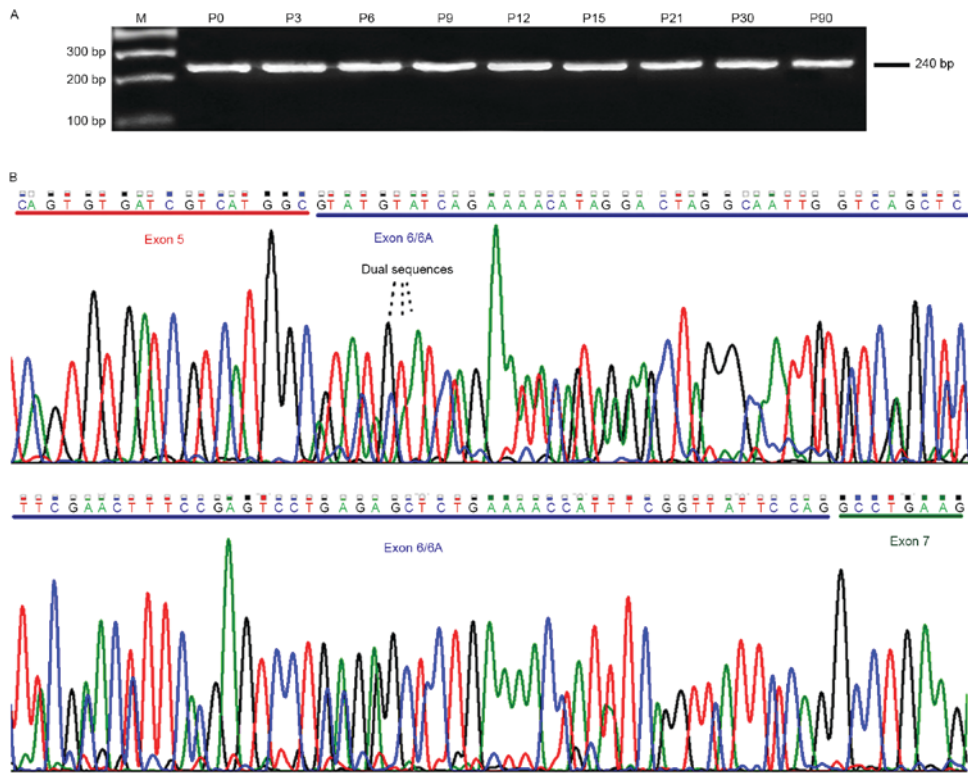


Figure 1. RT-PCR and DNA sequencing results with primer pair 1 targeting exon 6/6A of rat Na voltage-gated channel α subunit 5 gene. (A) RT-PCR results with primer pair 1. Electrophoresis revealed a single band at the expected size of 240 bp on the agarose gel. (B) Direct DNA sequencing results. Single sequence presented in the exon 5 or exon 7 coding regions, however a dual sequence appeared in the exon 6 coding region. DNA sequence analysis demonstrated that exon 6 and exon 6A were expressed in the frontal lobe of the rat brain. M, Marker; P, postnatal day; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs.

the PCR products, as a specific restriction enzyme site of *SacI* was observed to be present in exon 6A and not in exon 6. As indicated in Fig. 2A, three bands appeared on the agarose gel following enzyme digestion. The results indicated that PCR products including exon 6A were digested into two fragments whereas those containing exon 6 were preserved. Therefore, the expression ratio of neonatal Nav1.5 (Nav1.5e) vs. total Nav1.5 was detected from the signal quantification of pre- and post-digestion by autoradiography. In order to investigate the expression pattern of neonatal Nav1.5 in the rat brain with age development, frontal lobes of SD rats from P0-90 were excised. The results suggested that the expression ratio of neonatal Nav1.5 vs. adult Nav1.5 in the rat frontal lobe decreased from 1:1 to 1:3 with age development from P0-90 (Fig. 2B).

For comparison, the present study additionally detected the expression of neonatal Nav1.5 in ventricular myocytes of the rat heart from P0-90 using the aforementioned procedure. Notably, the DNA sequencing and restriction enzyme digestion results demonstrated the expression of neonatal and adult Nav1.5 in the ventricular myocytes of rat heart; however, the expression ratio of neonatal Nav1.5 vs. adult Nav1.5 mRNA decreased from a ratio of 1:4 to 1:19 in the ventricular myocytes of rat heart from p0-90, which was markedly different from that in the developing rat brain (Figs. 3 and 4). The results indicated that neonatal Nav1.5 mRNA expression was decreased in the ventricular myocytes of the rat heart during development.

Nav1.5a and *Nav1.5c* are expressed in the frontal lobe of rat brain and ventricular myocytes of rat heart. Primer pair 2,

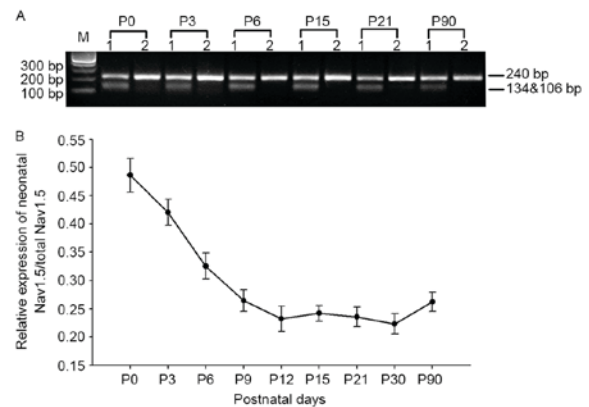


Figure 2. Expression pattern of neonatal and adult Nav1.5 splice variants in the frontal lobe of rat brain with age development. (A) Restriction enzyme digestion results. (B) Expression pattern of neonatal Nav1.5 in the developing rat brain. As indicated by the electrophoresis results, the expression ratio of neonatal Nav1.5 vs. adult Nav1.5 in the rat frontal lobe decreased from 1:1 to 1:3 with age development from P0-90. M, Marker; 1, polymerase chain reaction products with restriction enzyme *SacI*; 2, polymerase chain reaction products without restriction enzyme *SacI*; P, postnatal day; bp, base pairs; Nav1.5, voltage gated sodium channel 1.5.

targeting partial exon 17, full exon 18 and partial exon 19, were used to detect the expression of Nav1.5a and Nav1.5c in the frontal lobe of the rat brain. As indicated in Fig. 5, two bands with different sizes appeared on the agarose gel. Fragments of the two bands were extracted and sequenced. It was suggested that the longer fragment represented the wild-type Nav1.5

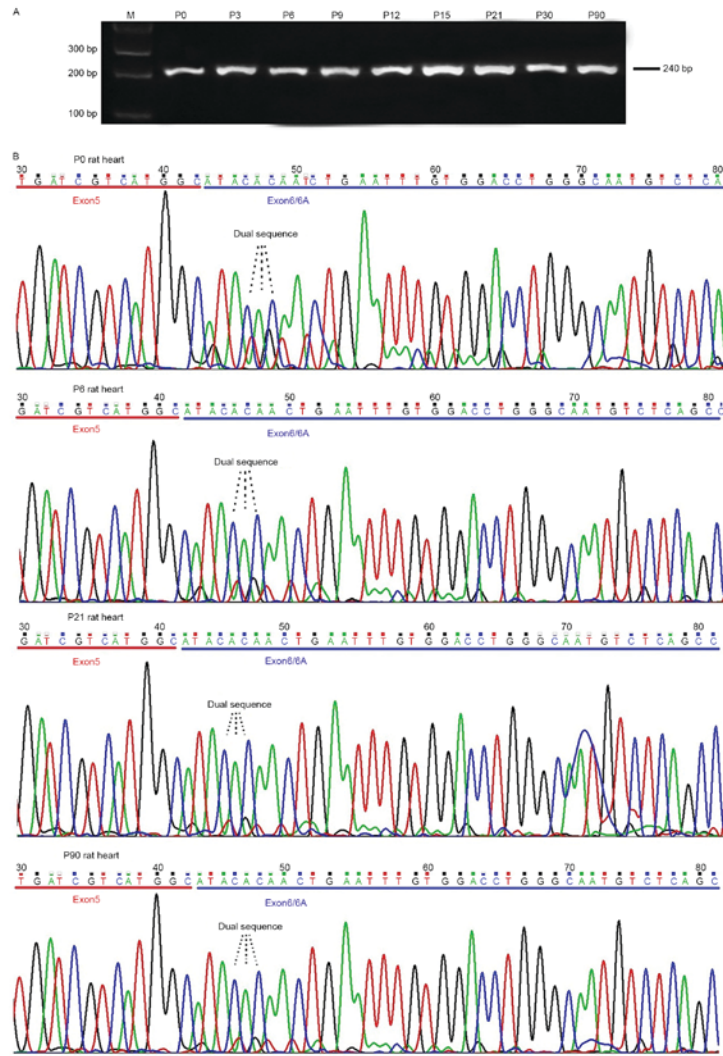


Figure 3. RT-PCR and DNA sequencing results with primer pair 1 in the rat heart. (A) RT-PCR results with primer pair 1. Electrophoresis results demonstrated a single band at the expected size of 240 bp on the agarose gel. (B) Direct DNA sequencing results. Single sequence presented in the exon 5 or exon 7 coding regions, however a dual sequence appeared in the exon 6 coding region. DNA sequence analysis demonstrated that exon 6 and exon 6A were expressed in the rat heart, however the expression level of exon6A decreased with age development from P0 to 90, determined from the peak height of exon6A in the DNA sequencing peak chart. M, Marker; P, postnatal day; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs.

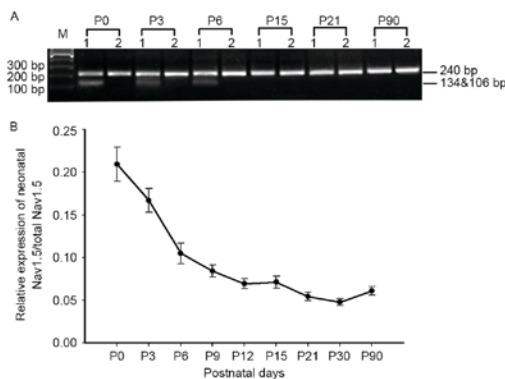


Figure 4. The expression pattern of neonatal and adult Nav1.5 splice variants in the ventricular myocytes of rat heart with age development. (A) Restriction enzyme digestion results. (B) Expression pattern of neonatal Nav1.5 in the developing rat heart. The expression ratio of neonatal Nav1.5 vs. adult Nav1.5 mRNA was 1:4 to 1:19 in the ventricular myocytes of rat heart from P0 to 90. M, Marker; P, postnatal day; 1, polymerase chain reaction products with restriction enzyme Sac I; 2, polymerase chain reaction products without restriction enzyme Sac I; bp, base pairs; Nav1.5, voltage gated sodium channel 1.5.

isoform and the shorter band was the Nav1.5a splice variant, in which exon 18 was alternatively spliced. Signal quantification via autoradiography (Fig. 5), revealed that the expression ratio of wild-type Nav1.5 compared with Nav1.5a was appropriately 1:4.5 and 1:1 in the neonatal (P0) and adult (P90) rat brain cortex of the frontal lobe, respectively. The results indicated that the expression ratio of wild-type Nav1.5 compared with Nav1.5a in the frontal lobe of the rat brain increased with age.

For comparison, the expression levels of Nav1.5a and wild-type Nav1.5 in the ventricular myocytes of rat heart from P0-90 were additionally detected (Fig. 6). The expression pattern of wild-type Nav1.5 compared with Nav1.5a was similar to that observed in the frontal lobe of rat brain with age development, however the expression ratio of the two splice variants was different in the two distinct tissue types. In the adult rat brain, wild-type Nav1.5 and Nav1.5a were observed to be present at a similar abundance. However, the expression ratio of wild-type Nav1.5 compared with Nav1.5a was appropriately 1:1 and 3.5:1 in the neonatal (P0) and adult (P90) rat heart, respectively, indicating that wild-type Nav1.5

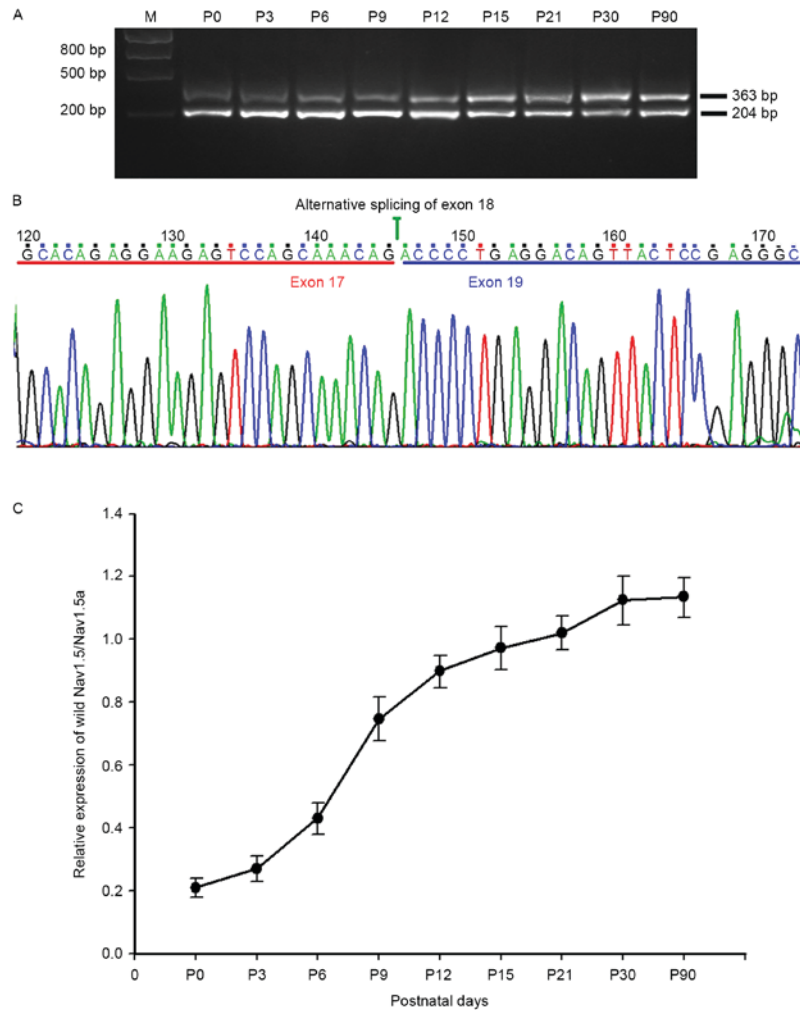


Figure 5. Expression pattern of Nav1.5a splice variant in the frontal lobe of rat brain with age development. (A) Reverse transcription-polymerase chain reaction results with primer pair 2. As indicated, two bands at the expected sizes of 363 and 204 bp were observed on the agarose gel. (B) DNA sequencing results. Fragments of the two bands were extracted and sequenced, respectively. The DNA sequencing results suggested that the longer fragment represented the wild-type Nav1.5 isoform while the shorter band was the Nav1.5a splice variant, in which exon 18 was alternatively spliced. (C) The expression pattern of Nav1.5a in the developing rat brain. The expression ratio of wild-type Nav1.5 vs. Nav1.5a was appropriately 1:4.5 and 1:1 in the neonatal (P0) and adult (P90) rat brain cortex of the frontal lobe, respectively. M, marker; P, postnatal day; bp, base pairs; Nav1.5, voltage gated sodium channel 1.5.

was the primary isoform expressed in the adult rat ventricular myocytes. The results demonstrated that although the expression of Nav1.5a mRNA reduced in the rat brain and heart, its expression quantification differed in the two tissue types.

DNA sequence analysis revealed that Nav1.5c (additional CAG codon, Q1077) was not detected in the frontal lobe of the rat brain and cardiac muscle in this experiment, indicating Nav1.5c may not be expressed, or is expressed at a very low level in these two tissue types.

Nav1.5d is not expressed in the frontal lobe of the rat brain. In order to detect if Nav1.5d, the splice variant with partial splicing of exon 17 (120 bp deletion in the intermediate region) was expressed in the frontal lobe of rat brain, primer P3 targeting the full length of exon 17 was used in PCR. The results demonstrated that only a single band with the expected size of 373 bp and not 253 bp was observed on the agarose gel (Fig. 7). Direct DNA sequencing further confirmed the inclusive expression of the full length exon 17 of SCN5A.

This result indicated that Nav1.5d may not be expressed or is expressed at a very low level in the frontal lobe of the rat brain.

Total Nav1.5 protein is expressed in the frontal lobe of the rat brain. In order to investigate if the Nav1.5 protein was expressed in the frontal lobe of rat brain, immunohistochemical and western blotting analyses were used to detect the expression and distribution of total Nav1.5 protein, in the neurons and glia cells of the frontal lobe of SD rat brain. The immunohistochemical results demonstrated that Nav1.5 protein was detected in the cortex of the frontal lobe, with the immunoreactivity predominantly observed in the neuronal cell bodies and processes, including axons and dendrites, whereas little or no immunoreactivity was detected in the glial components (Fig. 8). Notably, pyramid cells in layer V of the gray matter of the frontal lobe cortex demonstrated a greater level of immunoreactivity to the Nav1.5 antibody, compared with neurons in other layers (Fig. 8). Western blot analyses confirmed the expression of total Nav1.5 protein in the frontal

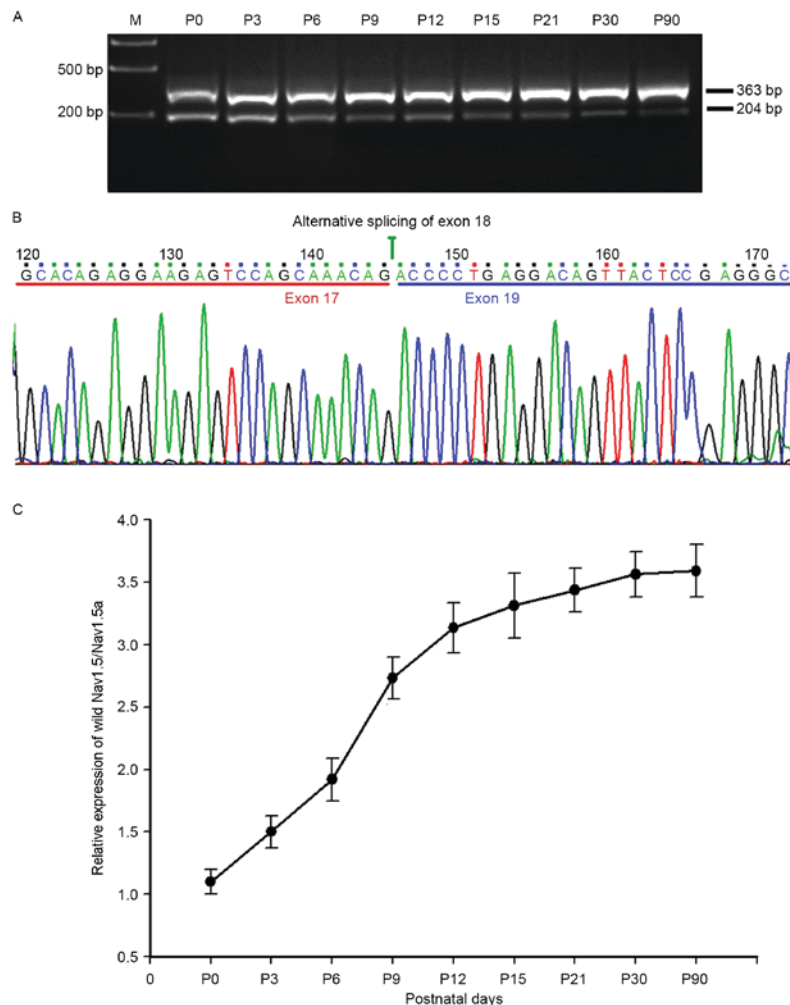


Figure 6. Expression pattern of Nav1.5a splice variant in the ventricular myocytes of rat heart with age development. (A) Reverse transcription-polymerase chain reaction results with primer pair 2. Two bands at the expected size of 363 bp and 204 bp were observed on the agarose gel. (B) DNA sequencing results. DNA sequencing results demonstrated that the longer fragment represented the wild-type Nav1.5 isoform and the shorter band the Nav1.5a splice variant. (C) The expression pattern of Nav1.5a in the developing rat heart. The expression ratio of wild-type Nav1.5 vs. Nav1.5a was appropriately 1:1 and 3.5:1 in the neonatal (P0) and adult (P90) rat ventricular myocytes, respectively. M, marker; P, postnatal day; bp, base pairs; Nav1.5, voltage gated sodium channel 1.5.

lobe of SD rats (Fig. 9). These results confirmed the expression and distribution of Nav1.5 in the frontal lobe cortex of rat brain.

Electrophysiological properties of Nav1.5 in the rat brain:

TTX-R current isolated from layer V pyramidal neurons of the frontal lobe. Whole-cell patch-clamp recordings were conducted on layer V pyramidal neurons of the frontal lobe to confirm if Nav1.5 was functionally expressed in the neurons. In order to isolate the Na current from the total current, CdCl₂ (200 μM) and TEA (20 mM) were added to the bath solution to block Ca²⁺ and K⁺ currents, respectively. To isolate the TTX-R Na current from the total Na current, a protocol specific to obtaining the total and TTX-R Na currents was used (35). TTX was applied at differing concentrations (10, 100 and 300 nM, and 1 μM) to record the Na current via whole-cell patch clamping. As indicated in Fig. 10, the TTX-R current was isolated and recorded in accordance with the aforementioned protocol. The TTX-R Na current was activated at -40 mV and reached the maximum amplitude at 0 mV. To further confirm the results, TTX was added to the bath solution. As presented

in Fig. 11, the Na current was recorded at 300 nM TTX, a concentration at which the TTX-S Na current was completely blocked. When the TTX concentration was increased to 1 μM, the Na current was not detected.

Discussion

Alternative splicing in Nav genes may generate structurally and functionally distinct Na channels (33,34,36,37). The authors previously demonstrated that Nav1.5a, Nav1.5e and Nav1.5f splicing variants were expressed in the rat brain (9,10,13). The present study systematically investigated the expression of further Nav1.5 splice variants in the frontal lobe of rat brain. The expression pattern of Nav1.5 splice variants with age development was additionally detected in this experiment. RT-PCR and DNA sequencing confirmed the expression of neonatal and adult Nav1.5 isoforms in the frontal lobe of the rat brain at different developmental stages. However, the expression level of neonatal Nav1.5 decreased with age development. Specifically, the expression of neonatal Nav1.5 mRNA compared with adult Nav1.5 mRNA decreased from

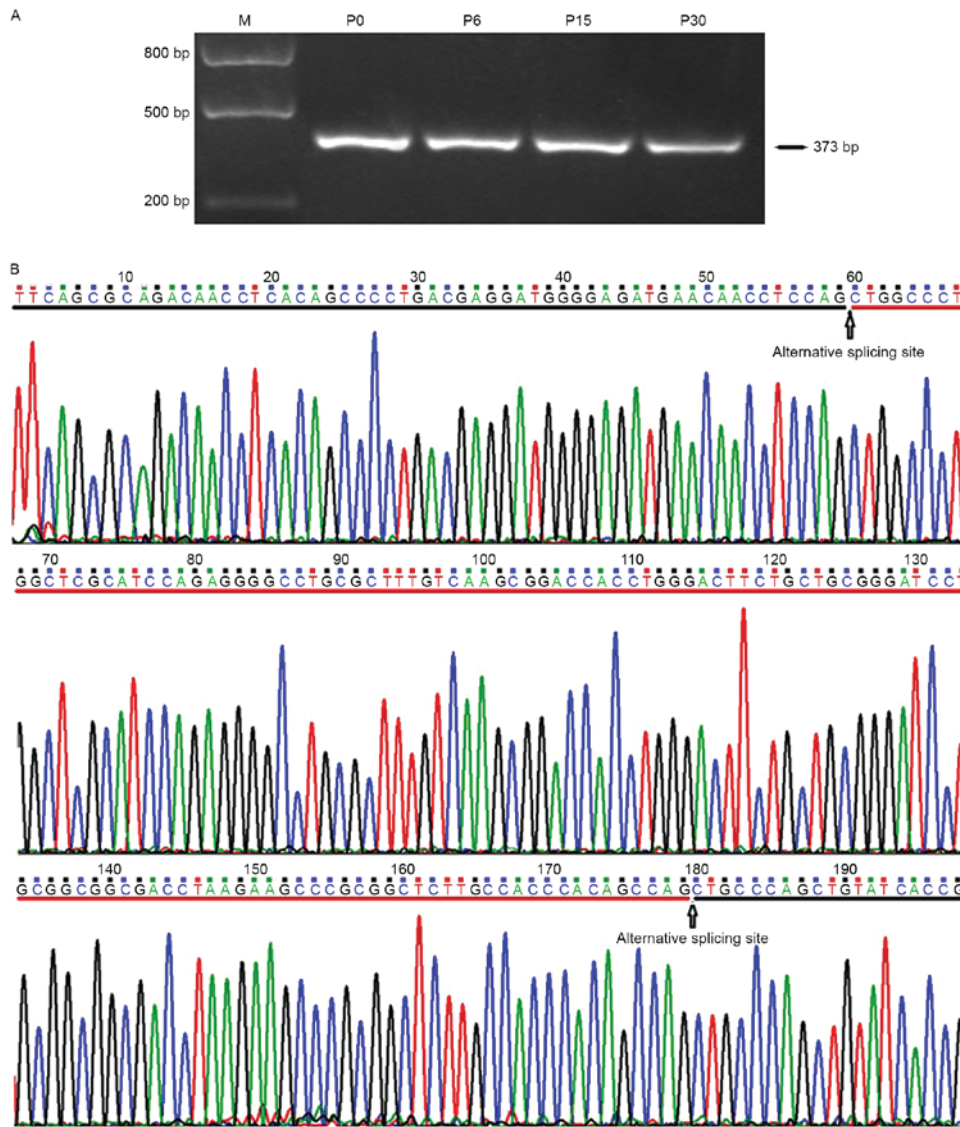


Figure 7. PCR and DNA sequencing results with primer P3 targeting Nav1.5d. (A) PCR results with primer P3. Electrophoresis results revealed the presence of one band at the expected size of 373 bp and not 253 bp on the agarose gel. (B) DNA sequencing results. Direct DNA sequencing of the PCR products confirmed the inclusive expression of the full length exon 17. M: marker; P, postnatal day; PCR, polymerase chain reaction; bp, base pairs; Nav1.5, voltage gated sodium channel 1.5.

postnatal day 0 (1:1) to 9 (1:3) and appeared to be constant from P12 to 90 in the frontal lobe of the rat brain, which indicated a reducing expression pattern of neonatal Nav1.5 with age development. The expression pattern of neonatal Nav1.5 splice variants with age development is similar to that of various other Navs, including Nav1.3 (38). However, as the 'neonatal' isoform of Nav1.5 may be detected in the adult rat brain cortex and the 'adult' Nav1.5 isoform is expressed in the neonatal rat brain cortex, the terms 'neonatal' and 'adult' are commonly used and do not necessarily denote the strict expression of the neonatal isoform to neonates and the adult isoform to adults. Therefore, the present study, in accordance with previous studies, selected the terminology 'Nav1.5e' rather than 'neonatal Nav1.5' to describe this splicing variant in normal tissues. Conversely, numerous authors decide to use the terminology 'neonatal Nav1.5' when investigating the expression of Nav1.5 in tumor cell lines (8,23,27,29,32), as 'neonatal' is there used to represent the re-expression of an

embryonic gene or oncogene and its expression is associated with the occurrence and development of tumors.

The Nav1.5a splicing variant, with the alternative splicing of exon 18 of SCN5A gene, was additionally detected in this investigation. The expression ratio of Nav1.5a compared with wild-type Nav1.5 altered with age development. In the neonatal rat brain cortex (P0), the expression ratio of these two variants was 4.5:1; however, in the adult rat brain cortex, the expression ratio was 1:1. The results indicated that wild-type Nav1.5 and Nav1.5a were expressed in the adult rat brain cortex and presented with a similar abundance, which was consistent with previous studies (7,13). Similar expression patterns of Nav1.5a were observed in the human neuroblastoma cell line NB-1 (22). However, Nav1.5a was not detected in the human brain cortex, indicating that the Nav1.5a transcript may be specific only for small rodents or certain tumor cell lines (10). The electrophysiological properties of wild-type Nav1.5 and Nav1.5a were similar to those indicated in the author's previous

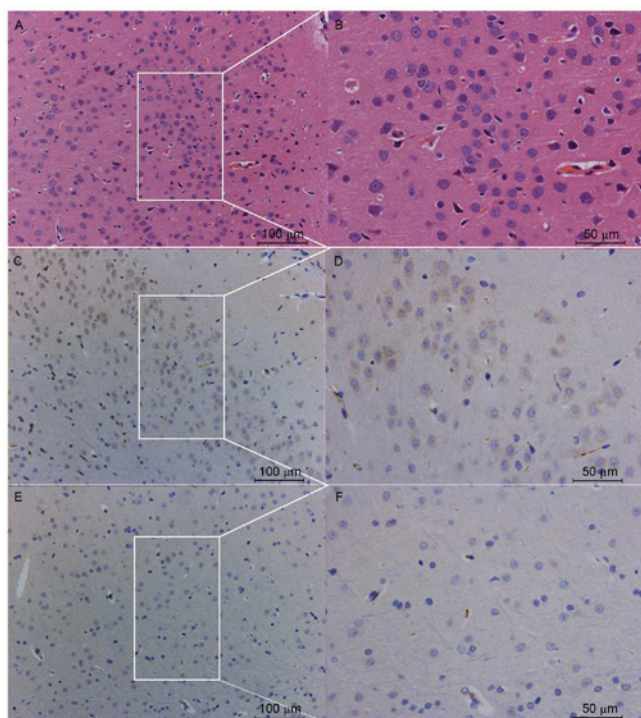


Figure 8. Immunohistochemical results of Nav1.5 protein in the frontal lobe of rat brain. Hematoxylin and eosin staining of cortex of rat brain at (A) 100 μm and (B) 50 μm . Nav1.5 staining (dark brown) in the gray matter of rat brain cortex at (C) 100 μm and (D) 50 μm . Nav1.5 staining in the white matter at (E) 100 μm and (F) 50 μm . Nav1.5 immunoreactivity was predominantly observed in the neuronal processes and cell bodies, including axons and dendrites, whereas little or no immunoreactivity was detected in the glial components. Scale bar, 100 μm for A, C and E and 50 μm for B, D and F. Nav1.5, voltage gated sodium channel 1.5.

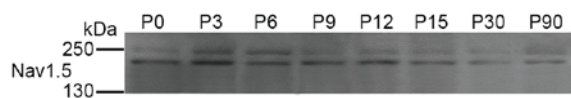


Figure 9. Western blotting results of Nav1.5 protein in the frontal lobe of rat brain. Western blotting on total protein extracts (100 μg protein/lane) from the frontal lobe confirmed the expression of Nav1.5 protein in the rat brain. The molecular weight markers indicated that a band of ~ 220 kDa was observed from Postnatal day 0 to P90. Nav1.5, voltage gated sodium channel 1.5.

study of Nav1.5a cloned from the human neuroblastoma cell line NB-1 (22). However, they were different in the kinetics of steady-state inactivation and activation, indicating the alternative splicing of exon 18 did alter the electrophysiological properties of Nav1.5.

The splicing variant Nav1.5c, characterized by an additional CAG trinucleotide (encoding an additional glutamine at position 1077) at the starting site of exon 18, was not detected in the frontal lobe of the rat brain in the present study, which was concordant with the author's previous study (13). Notably, Nav1.5c has been detected in the human brain cortex (10), with the expression ratio of Nav1.5c vs. wild-type Nav1.5 at 1:5, indicating the differing expression patterns of Nav1.5 splice variants in different species. The electrophysiological properties of the Nav1.5c variant were indistinguishable to wild-type Nav1.5 under normal physiological conditions, however the

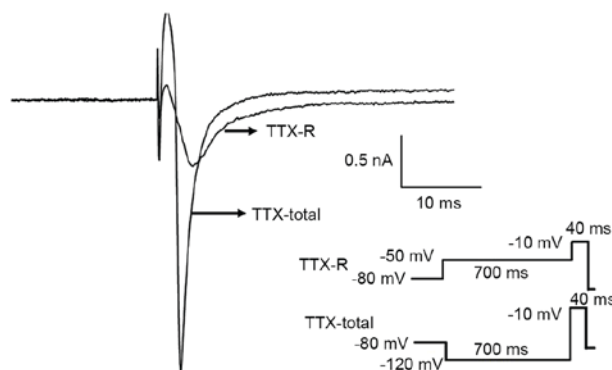


Figure 10. TTX-R current recorded in the layer V pyramidal neurons of the frontal lobe. The total Na^+ current was recorded with a 700 ms prepulse to -120 mV followed by the test pulse. The TTX-R Na^+ current was recorded with a 700 ms prepulse to -50 mV followed by a test pulse. The holding potential was at -80 mV. All the test pulses were 40 ms to -10 mV pulses. R, resistant; TTX, tetrodotoxin.

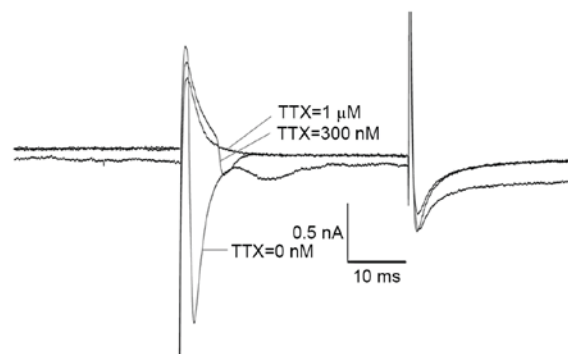


Figure 11. Isolation of TTX-R current recorded in the layer V pyramidal neurons of the frontal lobe via TTX. In order to isolate the TTX-R Na^+ current from the total Na^+ current, differing concentrations of TTX (10 nM, 100 nM, 300 nM and 1 μM) were added to the solutions. CdCl_2 (200 μM) and tetraethylammonium (20 mM) were added to the bath solution to block Ca^{2+} and K^+ currents, respectively. As indicated, the Na^+ current was recorded when the TTX concentration was 300 nM and TTX-S Na^+ current was blocked completely. However, when the TTX concentration was elevated to 1 μM , the Na^+ current disappeared completely. This value of TTX-sensitivity was consistent with those reported for other Nav1.5 clones (0.3-10 μM) and lower than those reported for Nav1.8 and Nav1.9 (>40 μM) channels, indicating that the TTX-R Na^+ current recorded in the neurons of frontal lobe of rat brain was generated by Nav1.5. R, resistant; TTX, tetrodotoxin; S, sensitive; Nav, voltage gated sodium channel; tetraethylammonium.

Nav1.5 kinetics alter significantly when expressing various mutations in the wild-type Nav1.5 (39-41).

Overall, with the exception of Nav1.5c and Nav1.5d, adult Nav1.5 and various Nav1.5 splice variants, including Nav1.5a, Nav1.5e and Nav1.5f, were expressed in the frontal lobe of the rat brain.

Nav1.5 is the primary cardiac Na^+ channel as it demonstrates the greatest expression in the heart and is important in the generation and propagation of action potentials in the electrophysiological activities of cardiac tissues (1,33). Previous studies suggest that neonatal Nav1.5 is not detected in the adult mouse, rat and human hearts (8,42,43). The present study systematically investigated the expression of adult and neonatal Nav1.5 in the rat ventricular myocytes with age development via RT-PCR, DNA sequencing and restriction enzyme

digestion methods. DNA sequencing of the PCR products indicated that neonatal and adult Nav1.5 variants were expressed in the rat ventricular myocytes of P0 to 90. However, the expression level of neonatal Nav1.5 was low in adult rat ventricular myocytes compared with that in the neonatal rat heart. Direct DNA sequencing of the PCR products from the rat ventricular myocytes revealed the dual sequences in the exon 6/6A coding region of SCN5A gene in neonatal and adult rat heart, however the additional band generated by the enzyme digestion was only observed in the neonatal rat ventricular myocytes and not in the adult, which further confirmed the low expression level of neonatal Nav1.5 in the adult rat ventricular myocytes (undetectable level by electrophoresis on the agarose gel). As indicated by electrophoresis results following enzyme digestion, the expression level of neonatal Nav1.5 reduced with age development from p0 to 6. The band signal on the agarose gel suggested that the expression ratio of neonatal Nav1.5 vs. adult Nav1.5 decreased from 1:4 to 1:19 from P0 to 90. These results confirmed the adult Nav1.5 mRNA expressed in the adult rat heart accounted for >95% of total Nav1.5 mRNA whereas the neonatal Nav1.5 mRNA represented <5%. As the expression level of neonatal Nav1.5 mRNA is low, the neonatal Nav1.5 protein has previously been demonstrated to be undetectable in the adult heart (8).

The expression of Nav1.5a in the developing rat ventricular myocytes was additionally observed in the present study. The expression pattern of wild-type Nav1.5 compared with Nav1.5a was similar to that in the rat brain with age development, the expression ratio of the two splice variants was different in the two distinct tissue types. In the adult rat brain cortex, wild-type Nav1.5 and Nav1.5a were present at a similar abundance. However, in the adult (P30~90) rat ventricular myocytes, the expression quantification of wild-type Nav1.5 was ~3.5 fold compared with Nav1.5a, indicating the wild-type Nav1.5 was the major isoform in the adult rat heart. These results were similar to those observed in previous studies (7,44) and demonstrated that although the expression of Nav1.5a reduced in the rat heart and brain with age development, its expression quantification was different in these two tissue types.

Neonatal Nav1.5, adult Nav1.5 and Nav1.5a were all expressed in the rat brain cortex and cardiac muscle, however the expression ratios among these splice variants differed in the distinct tissue types. Further studies may explore the underlying mechanisms regulating the alternative splicing of the SCN5A gene in the same or differing tissue types with age development.

Previous studies have detected the TTX-insensitive or resistant heart-like Na (Nav1.5) current in the striatal, hippocampal, medial entorhinal and olfactory sensory neurons of the rat brain (3,14,45,46). The present study recorded the Na current from the pyramidal cells in layer V of the frontal lobe of the SD rat brain via the whole-cell patch clamp technique. As K⁺ and Ca²⁺ currents may be recorded simultaneously, TEA and CdCl₂ were added to the bath solution in order to respectively block them. Various Na channel types, including Nav1.1, Nav1.2, Nav1.3, Nav1.5 and Nav1.6, may be co-expressed in brain neurons, therefore, the Na currents recorded in this experiment were compound products, including the TTX-S and TTX-R Na currents. In order to isolate the TTX-R Na

current from the total Na current, different concentrations of TTX were added to the bath solution. In accordance with previous results, the TTX-R Na current was detected in the neurons of the frontal lobe of the rat brain. However, two independent approaches were used to distinguish the TTX-S and TTX-R Na currents in the present study. The TTX-R Na current was recorded in 300 nM TTX, in which TTX-S Na channels were blocked completely. The TTX-R Na current disappeared when the concentration of TTX was increased to 1 μM. This value of TTX-sensitivity was consistent with those reported for other Nav1.5 variants (0.3-10 μM) and was lower than those reported for Nav1.8 and Nav1.9 (>40 μM) channels, indicating that the TTX-R Na current recorded in the neurons of the frontal lobe of the rat brain was generated by Na channel Nav1.5 (1,3,14,37). Further studies are necessary in order to clarify the specific contributions of each Nav1.5 isoform to the total Nav1.5 current and the generation of action potential in neurons.

In conclusion, the results of the present study demonstrated that various Nav1.5 isoforms, particularly the neonatal and adult isoforms, were expressed in the rat brain, however their expression ratios varied. The electrophysiological analysis conducted using the whole-cell patch clamp technique further confirmed the functional expression of Nav1.5 in the brain neurons.

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