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NOVEL mRNA ISOFORMS OF THE SODIUM CHANNELS $Na_v1.2$, $Na_v1.3$ AND $Na_v1.7$ ENCODE PREDICTED TWO-DOMAIN, TRUNCATED PROTEINS

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Abstract

The expression of voltage-gated sodium channels is regulated at multiple levels, and in this study we addressed the potential for alternative splicing of the $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ mRNAs. We isolated novel mRNA isoforms of $Na_v1.2$ and $Na_v1.3$ from adult mouse and rat dorsal root ganglia (DRG), $Na_v1.3$ and $Na_v1.7$ from adult mouse brain, and $Na_v1.7$ from neonatal rat brain. These alternatively spliced isoforms introduce an additional exon ($Na_v1.2$ exon 17A and topologically equivalent $Na_v1.7$ exon 16A) or exon pair ($Na_v1.3$ exons 17A and 17B) that contain an in-frame stop codon and result in predicted two-domain, truncated proteins. The mouse and rat orthologous exon sequences are highly conserved (94-100% identities), as are the paralogous $Na_v1.2$ and $Na_v1.3$ exons (93% identity in mouse) to which the $Na_v1.7$ exon has only 60% identity. Previously, $Na_v1.3$ mRNA has been shown to be upregulated in rat DRG following peripheral nerve injury, unlike the downregulation of all other sodium channel transcripts. Here we show that the expression of $Na_v1.3$ mRNA containing exons 17A and 17B is unchanged in mouse following peripheral nerve injury (axotomy), whereas total $Na_v1.3$ mRNA expression is upregulated by 33% (P=0.003), suggesting differential regulation of the alternatively spliced transcripts. The alternatively spliced rodent exon sequences are highly conserved in both the human and chicken genomes, with 77-89% and 72-76% identities to mouse, respectively. The widespread conservation of these sequences strongly suggests an additional level of regulation in the expression of these channels, that is also tissue-specific.

Keywords

DRG; brain; alternative splicing; Scn2a; Scn3a; Scn9a

Voltage-gated sodium channels mediate the rapid influx of sodium ions that initiate action potentials in excitable cells. The nine mammalian pore-forming α-subunits $Na_v1.1$-$Na_v1.9$ each contain four internally homologous domains (I-IV) that are connected by the three interdomain cytoplasmic loops IDI/II, IDII/III and the smaller IDIII/IV (Plummer and Meisler, 1999; Goldin et al., 2000). Among these channels, the $Na_v1.5/1.8/1.9$ subfamily is resistant to the neurotoxin tetrodotoxin (TTX), whereas the other sodium channels are blocked by nanomolar concentrations and are considered to be TTX-sensitive (Plummer and Meisler, 1999).

The expression of mammalian voltage-gated sodium channels can be regulated at multiple levels, including transcription from multiple promoters (Drews et al., 2005; Shang and

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Dudley, 2005; Martin et al., 2007), alternative splicing of pre-mRNA and different levels of post-translational glycosylation (Diss et al., 2004). One of the best characterized examples of alternative splicing is that of $Na_v1.2$ and $Na_v1.3$ mRNAs which involves the inclusion of either an upstream exon 5N or downstream exon 5A, which encode part of domain I and differ by one or two residues in the 30 encoded amino acids (Sarao et al., 1991; Gustafson et al., 1993; Raymond et al., 2004). In the case of $Na_v1.2$, biophysical differences between the 5N and 5A isoforms have been demonstrated by heterologous expression in HEK293 cells (Xu et al., 2007). $Na_v1.2$ and $Na_v1.3$ transcripts containing exon 5N are predominant in fetal and neonatal brain, whereas in adult brain the exon 5A transcript is predominant (Sarao et al., 1991; Gustafson et al., 1993). Similar brain expression patterns have also been described for $Na_v1.6$ in the adult ((Plummer et al., 1998; Raymond et al., 2004); coding exon 5) and for $Na_v1.7$ in the fetus (Raymond et al., 2004). Interestingly, there is also alternative splicing of exons that encode the corresponding transmembrane segments of $Na_v1.6$ domain III, but in this case the alternative downstream exon 18A encodes 41 amino acids whereas the upstream exon 18N contains an in-frame stop codon that results in the 18N isoform encoding a predicted two-domain protein i.e. a truncated protein that lack domains III and IV (Plummer et al., 1997). Such a truncated protein would not function as a sodium channel (Cox et al., 2006). $Na_v1.6$ mRNA containing exon 18N is the major isoform in fetal brain and is downregulated postnatally, whereas the 18A isoform is expressed in fetal brain and becomes predominant postnatally (Plummer et al., 1997).

We have previously characterized alternatively spliced mRNA isoforms encoding the IDII/III cytoplasmic loop of $Na_v1.8$ in adult dorsal root ganglia (DRG) (Kerr et al., 2004) and of the more widely expressed $Na_v1.5$ in adult DRG, brain and heart (Kerr et al., 2004, 2007). In contrast, no alternative splicing of $Na_v1.9$ mRNA was detected in DRG (Kerr et al., 2004). Here, we extend these studies to the TTX-sensitive channels and describe the isolation of novel, conserved mRNA isoforms of $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ expressed in DRG or brain.

Part of the mouse data has been presented previously in abstract form (#622.8, Society for Neuroscience meeting, 2005; http://www.sfn.org).

EXPERIMENTAL PROCEDURES

Animals, tissue collection, RNA extraction and reverse transcription (RT)

All animals were fed standard chow and water *ad libitum*, and all procedures were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. All reasonable efforts were made to minimize animal suffering and to use the minimum number of animals necessary to perform statistically valid analyses.

Tissues from 11 week-old male C57BL/6J mice (Bristol University colony, Bristol, UK) and from neonatal (postnatal day 1) or adult male (~300 g) Wistar rats (Bristol University colony) were frozen on dry ice and stored at -80 °C. Total RNA isolation, DNase treatment and re-extraction, and RT reactions with random hexamers were as previously published (Kerr et al., 2004).

Studies on peripheral nerve transection (axotomy) were exactly as previously published (Kerr et al., 2004, 2007), with the right sciatic nerve of 10-12 week-old male mice (Bristol University colony) transected at the mid-thigh level, prior to killing 7 days later by cervical dislocation to obtain ipsilateral (axotomized) and contralateral (control) lumbar L4 and L5 DRG pools each from nine animals.

Cloning of $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ partial-length cDNAs

Partial-length cDNAs were each amplified by RT-PCR of 5 μl RT reaction (100 ng of total RNA equivalent) with HPLC-purified primers (Invitrogen, Paisley, Strathclyde, UK; sequences detailed below) for 40 cycles, using previously described PCR conditions (Kerr et al., 2007) except for annealing at 66 °C. Products were excised from ethidium bromide-stained agarose gels (imaged in inverted contrast), purified and TA-cloned into pCRII-TOPO (Invitrogen), all as in Kerr et al. (2007). DNA sequencing was performed by the Department of Biochemistry, Oxford University, now Geneservice.

Mouse $Na_v1.2$ forward (5′-GATACGTGAATTCATTCAGAAAGCC-3′) and reverse (5′-GGTTTTCCTCAAGTTCCACCAGAG-3′) primers correspond, respectively, to nucleotides (nt) 159-183 of a mouse anonymous partial-length expressed sequence tag (CF723518) and nt 10,384-10,361 of an anonymous genomic DNA clone (BX284648), each identified using the rat cDNA sequence (NM_012647) (Noda et al., 1986). The expected product spans exons corresponding to human *SCN2A* exons 17-19 (Kasai et al., 2001). All sequenced clones contained an A residue consistent with nt 8315 of BX284648 (and recent genomic-derived XM_001001618), rather than the corresponding C residue at nt 567 of CF723518.

Mouse $Na_v1.3$ forward (5′-TACGGGAGTGCTTCCGAAAAGCG-3′) and reverse (5′-AGGTCTTCCTAAGATTCCACCAGAT-3′) primers correspond, respectively, to nt 137,831-137,809 and nt 125,694-125,718 of an anonymous genomic DNA clone (AL928621), with the expected product spanning exons corresponding to human *SCN3A* exons 17-19 (Kasai et al., 2001).

Mouse $Na_v1.6$ forward (5-GGGCCAAAGTGAAGGTGCATGCC-3′) and reverse (5′-GCCTAGTCCTTCCTCGATGTTGAC-3′) primers correspond, respectively, to nt 3023-3045 and 3537-3514 of the published cDNA sequence (AF049617) (Smith et al., 1998). Spanned sequence corresponds to human *SCN8A* exons 14-16 (Plummer et al., 1998), topologically equivalent to *SCN2A* and *SCN3A* exons 17-19 (Kasai et al., 2001). All sequenced cDNA clones from C57BL/6J (n=24) and 129/OlaHsd (n=10) mouse strains contained the silent nucleotide substitution T3360C, which is also present in an anonymous genomic DNA clone (AC104834, nt 72,387) and recent cDNA sequences (NM_001077499, AK083220).

Mouse $Na_v1.7$ forward (5′-TGGCCAGAATTAAAAGAGGGATCAAT-3′) and reverse (5′-GGTCTTCCTGATGGTCCACCAAAC-3′) primers correspond, respectively, to nt 119,164-119,139 and 100,775-100,798 of a mouse anonymous genomic DNA clone (AL928726) identified using the rat cDNA sequence (NM_133289) (Sangameswaran et al., 1997). The expected product spans exons corresponding to human *SCN9A* exons 16-18 (exon numbering following (Yang et al., 2004; Cox et al., 2006)), equivalent to *SCN2A* and *SCN3A* exons 17-19 (Kasai et al., 2001).

Rat $Na_v1.2$ primers 5′-AGCCTTTGTCAGAAAGCAGAAAGCTTT-3′ and 5′-CTTATCTGACAACACTTGAACTTTCTC-3′ correspond, respectively, to nt 3291-3317 and 3764-3738 of NM_012647 (Noda et al., 1986). Rat $Na_v1.3$ primers 5′-GAAAAATAAGATACGGGAGTGCTTCCG-3′ and 5′-TTCTTCTGTACTTACTTGACAGAAGG-3′ correspond, respectively, to nt 3320-3346 and 3824-3799 of NM_013119 (Kayano et al., 1988). Rat $Na_v1.6$ primers 5′-GGACCAAAGTGAAGGTGCACGCCT-3′ and 5′-ACTTGCCTAGTCCTTCCTCGATGTTG-3′ correspond, respectively, to nt 3017-3040 and 3535-3510 of L39018 (Schaller et al., 1995) and share identity within NM_019266 (Dietrich et al., 1998). Rat $Na_v1.7$ primers 5′-GGAATCAATTACGTGAAACAGACCCT-3′ and 5′-

AACTTTCCCTTTCCCAGAGTCTACAT-3′ correspond, respectively, to nt 2989-3014 and 3525-3500 of NM_133289 (Sangameswaran et al., 1997).

Real-time quantitative RT-PCR assays

Real-time quantitative RT-PCR assays were used to derive relative mRNA expression levels by the comparative threshold cycle (C_t) method, as previously described along with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer and probe set (Kerr et al., 2007). Primer and probe sets (Applied Biosystems, Warrington, Cheshire, UK) for products of 75-150 base pairs (bp) were designed using Primer Express software (Applied Biosystems), and the probes detailed below had the 5′ fluorescent reporter dye FAM (6-carboxyfluorescein) and the 3′ quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine).

Primers and probe to detect the mouse $Na_v1.3$ mRNA isoform that includes exons 17A and 17B were: forward primer 5′-ACCCAG/TATCACAACTCTGGCAAT-3′ (where/denotes the exon 17A/17B junction, see Fig. 2B and C), reverse primer 5′-CAATTTCAGCTTGTTCACCTTCTC-3′ and Taqman probe 5′-AAATGAAA/AAATTAAATGCAACCAGCTCTTCTGAAG-3′ (where/denotes the exon 17B/18 junction, see Fig. 2C) that correspond, respectively, to nt (133,352-133,347+130,458-130,441), 126,965-126,988 and (130,438-130,431+127,043-127,016) of genomic DNA clone AL928621. Primers and probe to detect mouse 'total' $Na_v1.3$ mRNA were for a product that spanned intron 18 (1.15 kilobases (kb), AL928621/NT_039207): forward primer 5′-CGAGAAGGTGAACAAGCTGAAAT-3′, reverse primer 5′-TTTTTCCTTTACCTTCTTCCGTACTTAC-3′ and Taqman probe 5′-TGAAG/GATGCATTAAAAAATTTCCCTTCTGCC-3′ (where/denotes the exon 18/19 junction) that correspond, respectively, to nt 126,989-126,967, 125,718-125,745 and (126,927-126,923+125,774-125,748) of AL928621. The primer and probe set to detect mouse $Na_v1.6$ mRNA was designed using non-default Primer Express settings (maximum T_m difference=4 °C; maximum T_m=61 °C), for a product that spanned an intron of 386 nt (AC104834 nt 72,394-72,779) that is analogous to intron 14 of human *SCN8A* (Plummer et al., 1998): forward primer 5′-CCCCGAAGGCAGCAAAGACA-3′, reverse primer 5′-GCTCCACTGGGACTTCTTCCA-3′ and Taqman probe 5′-AGAAGGGAGTACCATCGACATCAAGCCTG-3′ that correspond, respectively, to nt 3348-3367, 3442-3422 and 3390-3418 of AF049617 (Smith et al., 1998). The underlined forward primer residue corresponds to the nucleotide substitution T3360C, as detailed in the previous section.

The specificity of each assay was demonstrated by testing against a panel of cDNA plasmids ($Na_v1.2$; $Na_v1.2$ (exon 17A); $Na_v1.3$; $Na_v1.3$ (exon 17A[83nt]+17B); $Na_v1.3$ (exon17A[92nt]+17B); $Na_v1.5$; $Na_v1.6$; $Na_v1.7$; $Na_v1.7$ (exon 16A); $Na_v1.8$; and $Na_v1.9$), and each assay amplified a single product of the expected size from DRG (data not shown). DRG expression levels were normalized to GAPDH mRNA, which is unchanged following peripheral nerve injury (Macdonald et al., 2001; Kerr et al., 2007), and results are presented as mean±S.E. of log-transformed data. The statistical significance of differences between control and axotomy samples was judged by two-tailed paired *t*-test, and between different genes after axotomy by two-way ANOVA with a Scheffé multiple comparison. $P<0.05$ was considered statistically significant.

Bioinformatics

The publicly available mouse (build 36, May 2006; strain C57BL/6J), rat (build 4, alternative assembly, July 2006; mixture of Brown Norway (BN) and Sprague-Dawley strains), human (build 36) and chicken (build 2.1) genomes were accessed using

megaBLAST and cross-species megaBLAST (http://ncbi.nlm.nih.gov/genome/seq/). The SECISearch 2.19 program (http://genome.unl.edu-SECISearch) was used to search for potential selenocysteine insertion sequences (Kryukov et al., 2003).

RESULTS

Expression of $Na_v1.2$, 1.3, 1.6 and 1.7 mRNAs in mouse DRG and brain

In order to study the expression of mouse $Na_v1.2$, 1.3, 1.6 and 1.7 mRNAs, gene-specific primers were designed to amplify across the three expected exons that encode the second (interdomain IDII/III) cytoplasmic loop (Plummer et al., 1998; Kasai et al., 2001). The interdomain cytoplasmic loops IDI/II and IDII/III are the least conserved regions between the different voltage-gated sodium channels (Schaller et al., 1995; Chen et al., 1997), and we have previously described alternatively spliced mRNA isoforms of $Na_v1.5$ and $Na_v1.8$ encoding the IDII/III cytoplasmic loop (Kerr et al., 2004, 2007).

In addition to $Na_v1.2$, 1.3, 1.6 and 1.7 RT-PCR products of the expected size (Fig. 1A) and sequence (each n=12), adult mouse DRG also expressed novel, minor isoforms of $Na_v1.2$ and $Na_v1.3$ mRNAs (Fig. 1A). The larger, minor $Na_v1.2$ product of 625 bp includes an additional 84 nucleotide (nt) region (n=24) in which the second codon is an in-frame stop codon (Fig. 2A), resulting in a predicted two-domain $Na_v1.2$ protein. This alternatively spliced sequence is transcribed from a previously unrecognized exon that we have designated 17A as it is present downstream of $Na_v1.2$ (*Scn2a* gene) exon 17 (Kasai et al., 2001). The larger, minor $Na_v1.3$ product of ~650 bp (Fig. 1A) contains either an additional 111 or 120 nt region, each with an in-frame stop codon (Figs. 2 and 3) and therefore encoding predicted two-domain $Na_v1.3$ proteins. Comparison of these novel sequences to genomic DNA shows that they are transcribed from two exons, here designated 17A (83 or 92 nt) and 17B (28 nt), with the 92 nt isoform of exon 17A (n=6/24) using a downstream donor splice site (Fig. 3; NT_039207). The $Na_v1.3$ (*Scn3a* gene) exon 17A sequence has 93% (78/84, gaps 1/84) nucleotide sequence identity to exon 17A of $Na_v1.2$ (*Scn2a*), but the relative deletion of a single nucleotide means that the open reading frame of $Na_v1.3$ continues to a termination codon in the second novel exon, exon 17B (Figs. 2 and 3). The previously described alternative splicing of $Na_v1.6$ that affects domain III (see introduction) would not be detected in this analysis of the region encoding the IDII/III cytoplasmic loop.

To address the possibility of tissue-specific expression of these novel mRNA isoforms, expression of each channel was also studied in adult mouse brain. In addition to major RT-PCR products of the expected size (Fig. 1B and C) and sequence (each n=12), larger $Na_v1.3$ and $Na_v1.7$ products were detected (Fig. 1B). As in DRG, the larger, minor $Na_v1.3$ product includes the novel exons 17A (83 nt, n=19; 92 nt, n=5) and 17B (Fig. 2). The larger, minor $Na_v1.7$ product of ~650 bp (n=24) contains an additional 81 nt region with the second codon being an in-frame stop codon (Fig. 2D), that is transcribed from an exon we have designated 16A (Fig. 3; NT_039207). This exon numbering follows that of the human $Na_v1.7$ (*SCN9A*) gene (Yang et al., 2004; Cox et al., 2006) and is topologically equivalent to exon 17A of the $Na_v1.2$ (*Scn2a*) and $Na_v1.3$ (*Scn3a*) genes (this work (Kasai et al., 2001)). However, the $Na_v1.7$ (*Scn9a*) exon 16A has only 60% nucleotide sequence identity to the corresponding $Na_v1.2$ and $Na_v1.3$ sequences (respectively, 51/84 with 3/84 gaps, and 50/84 with 4/84 gaps; Fig. 2E).

Expression of $Na_v1.3$ mRNA isoforms in mouse DRG after axotomy

Following peripheral nerve injury, $Na_v1.3$ mRNA levels have been reported to be upregulated ~twofold in rat DRG (Kim et al., 2001; Hains et al., 2004) whereas the other voltage-gated sodium channel transcripts are downregulated by ~40-80% (Kim et al., 2001,

2002; Raymond et al., 2004). To assess the effect of peripheral nerve transection (axotomy) on the expression of mouse $Na_v1.3$ mRNA isoforms, real-time quantitative RT-PCR assays were optimized for $Na_v1.3$ mRNA containing exons 17A and 17B (hereafter 17A+B), and for 'total' $Na_v1.3$ mRNA (i.e. detecting the isoforms both including and excluding exons 17A+B). Note that the presence of the $Na_v1.3$-specific exon 17B allowed the design of the specific assay to $Na_v1.3$ mRNA containing exons 17A+B that did not cross-react with other sodium channels (see Experimental Procedures). Specific assays for $Na_v1.3$ mRNA excluding exons 17A+B or for $Na_v1.2$ mRNA containing exon 17A could not be designed due to the high sequence similarity of the relevant region of the $Na_v1.1/Na_v1.2/Na_v1.3$ upstream exons (respective $Na_v1.2/1.3$, $Na_v1.2/1.1$ and $Na_v1.3/1.1$ nucleotide identities in the 3 window of 75 nt are 89.3%, 90.7% and 89.3%) and the high level of sequence conservation (93%) between exons 17A of $Na_v1.2$ (*Scn2a*) and $Na_v1.3$ (*Scn3a*). Control real-time RT-PCR assays were also optimized for $Na_v1.6$ and galanin, which have previously been shown to be, respectively, downregulated and upregulated in rat DRG after peripheral nerve injury (Macdonald et al., 2001; Kim et al., 2002; Raymond et al., 2004).

Seven days after axotomy, the expression of $Na_v1.3$ mRNA containing exons 17A+B was unchanged from control ($P>0.05$), whereas total $Na_v1.3$ mRNA increased by 33% compared with control ($P=0.003$) in mouse lumbar L4 and L5 DRG (Fig. 4). In addition, the effect of axotomy compared with control was significantly different between $Na_v1.3$ mRNA containing exons 17A+B and total $Na_v1.3$ mRNA ($P<0.001$, two-way ANOVA), suggesting that the alternatively spliced isoforms of $Na_v1.3$ mRNA are differentially regulated. The decrease in $Na_v1.6$ mRNA expression of 41% ($P<0.001$) after axotomy was similar to our previous results on $Na_v1.5$ and $Na_v1.8$ mRNAs (Kerr et al., 2007) and, as previously published in the rat ((Macdonald et al., 2001) and references therein), there was a massive induction of galanin mRNA expression following axotomy (83.6-fold; $P<0.001$).

Expression of $Na_v1.2$, 1.3, 1.6 and 1.7 mRNAs in rat DRG and brain

If the novel exons expressed in the mouse are biologically important, they would be expected to be conserved between species. As in the mouse, adult rat DRG expressed $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ mRNAs with RT-PCR products of the expected size and sequence (each $n=12$) along with novel, larger isoforms of $Na_v1.2$ and $Na_v1.3$ (Fig. 5A and B). Sequenced clones from the larger, minor $Na_v1.2$ product had an insertion of 84 nt ($n=16/18$), or 93 nt ($n=2/18$), that includes a conserved in-frame stop codon and has 95% (80/84) nucleotide sequence identity with the mouse exon 17A (Fig. 2A). Sequenced clones of the larger, minor $Na_v1.3$ isoform ($n=18$) each had a 112 nt insertion due to an exon 17A (84 nt) and exon 17B (28 nt). However, unlike the mouse $Na_v1.3$ (*Scn3a*) exon 17A sequence of 83 nt which starts with 5′-GGTGA-3′ and has an open reading frame extending into exon 17B, the rat exon 17A sequence starts with 5′-GGGTGA-3′ which includes an in-frame stop codon (underlined; Fig. 2B and C). The rat $Na_v1.3$ (*SCN3A*) exons 17A and 17B have, respectively, 94% (78/84, with gap 1/84) and 100% (28/28) nucleotide sequence identity with the orthologous mouse sequences, and the rat *SCN3A* exon 17A sequence is identical to that of *SCN2A* (Fig. 2).

Adult rat brain expressed $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ mRNAs, but no larger $Na_v1.3$ or $Na_v1.7$ mRNA isoforms equivalent to those found in mouse brain were detected (Fig. 5C and data not shown). To address whether an additional $Na_v1.7$ isoform was synthesized in rat tissue, neonatal brain was also screened. In addition to $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ products of the expected sizes and sequences (each $n=12$), a larger $Na_v1.7$ product was readily detected (Fig. 5C) with an insertion of 80 nt that includes an in-frame stop codon ($n=18$) and that has 94% (77/82, gaps 3/82) nucleotide sequence identity to the orthologous mouse exon 16A sequence (Fig. 2D).

The splice sites sequences of the alternatively spliced mouse and rat exons

As shown in Table 1, the novel *Scn2a* ($Na_v1.2$), *Scn3a* ($Na_v1.3$) and *Scn9a* ($Na_v1.7$) exons are each flanked by introns with the canonical GT and AG dinucleotides at the 5′ and 3′ splice sites, respectively, except for the mouse *Scn3a* exon 17A variant of 92 nt and rat *SCN2A* exon 17A variant of 93 nt that result from selecting a downstream donor splice site of a G**C**-AG intron (see Discussion). By comparison to the consensus mammalian 5′ splice site sequence **(C/A)AG**/GT(A/G)AGT (exon nt in bold; (Burset et al., 2001)), the conserved CAG/GT**T**A*CC* of mouse/rat *Scn2a* and *Scn3a* introns 17A and the corresponding *Scn9a* intron 16A each differ at the underlined positions +3, +5 and +6 (respective mammalian nucleotide frequencies: T=2.5%; C=5.5% and C=16.5%; (Burset et al., 2001)). In addition, they do not conform to the strong tendency for a thymine (T) residue at position +3 to occur along with a guanine (G) at position +5 (Clark and Thanaraj, 2002), though cytosine (C) residues at positions +5 and +6 are known to be more common in alternative cassette exons expressed in neurons (Stamm et al., 2000).

The rodent novel exon sequences are highly conserved in the human and chicken genomes

Human genomic sequences with high nucleotide sequence identity to the novel exons identified in mouse and rat were identified in silico in the corresponding locations of *SCN2A* (87% identity), *SCN3A* (exons 17A and 17B: respectively 85% and 89% identities) and *SCN9A* (77% identity) (Fig. 6A-D; NT_005403). In each case the putative exon is flanked by GT-AG introns (data not shown), and the presence of an in-frame stop codon predicts a two-domain isoform of each protein. The human putative exon 17A sequences of *SCN2A* and *SCN3A* are identical, as in the rat, and share 80% identity with exon 16A of *SCN9A* ($Na_v1.7$).

A useful outgroup to the mammals is provided by the chicken (*Gallus gallus*) genome sequence, in which three putative alternative exons were also identified (Fig. 6; NW_001471729). Within the chicken *SCN2A*/locus395945 gene an 85 nt sequence shares 72% and 80% identities with the mouse and human *Scn2a* exons 17A, respectively, and within the *SCN3A*/locus424180 gene an 84 nt sequence shares 76% and 86% identities with the mouse and human *Scn3a* exons 17A, though no corresponding *Scn3a* exon 17B sequence was detected (Fig. 6). The third putative exon of 84 nt, within a gene referred to here as *SCN9A* (see Discussion), shares 73% and 84% identities with the mouse and human *Scn9a* ($Na_v1.7$) exons 16A (Fig. 6D). Each of these chicken putative exons contains a conserved in-frame stop codon, and is flanked by GT-AG introns (Fig. 6; data not shown). The chicken putative *SCN2A* and *SCN3A* exons have 85% identity and, respectively, share 86% and 89% identities with the putative exon of *SCN9A* ($Na_v1.7$).

DISCUSSION

$Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ mRNAs were each detected in DRG and brain from adult mouse and rat (Figs. 1 and 5), as previously reported for rat (Schaller et al., 1995; Felts et al., 1997; Sangameswaran et al., 1997; Dietrich et al., 1998; Kim et al., 2001), mouse brain $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$ (Smith et al., 1998; Drews et al., 2005; Martin et al., 2007) and mouse DRG $Na_v1.6$ (Drews et al., 2005). In addition, we isolated novel mRNA isoforms of $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ that result from the inclusion of exon(s) that contain an in-frame stop codon, and therefore encode predicted two-domain proteins. These exons are highly conserved, with mouse-rat orthologous sequences sharing 94-100% nucleotide identity, and there is also conservation between the paralogous exons e.g. the mouse *Scn2a* and *Scn3a* exon 17A sequences share 93% identity, whereas they each have only 60%

identity to the corresponding *Scn9a* ($Na_v1.7$) exon 16A sequence (Fig. 2), consistent with their general phylogenetic relatedness (Plummer and Meisler, 1999; Novak et al., 2006a).

The alternatively spliced mRNA isoforms of $Na_v1.2$ and $Na_v1.3$ were expressed in both adult mouse and rat DRG, and alternatively spliced mRNA isoforms of $Na_v1.3$ and $Na_v1.7$ were expressed in adult mouse brain (Figs. 1 and 5). The sodium channel $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ mRNAs are known to be expressed in mouse heart (Haufe et al., 2005; Marionneau et al., 2005), but although we detected each channel in adult whole-heart, no additional isoforms were detected (data not shown). Therefore in mouse, each of the alternatively spliced isoforms is tissue-specific. Clearly, the regulation of the expression of these alternatively spliced $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ mRNAs is highly complex, with species differences between adult mouse and rat brain, and developmental differences between the neonate and adult in rat brain (Figs. 1 and 5). In addition, rare mRNA isoforms of rat $Na_v1.2$ and mouse $Na_v1.3$ include nine nucleotide extensions of exons 17A (Fig. 3) due to the selection of a downstream splice donor site, which creates G*C*-AG introns. These are the most common type of non-canonical splice site pair and are processed by the standard U2-type spliceosome (Wu and Krainer, 1999; Burset et al., 2000), and a similar alternatively spliced nine nucleotide extension that encodes part of the $Na_v1.3$ IDI/II cytoplasmic loop also creates a G*C*-AG intron (Kasai et al., 2001; Thimmapaya et al., 2005).

There has been much interest in $Na_v1.3$ expression following the finding that $Na_v1.3$ mRNA is the only sodium channel transcript that is upregulated in the rat DRG after nerve injury (Waxman et al., 1994; Dib-Hajj et al., 1996; Kim et al., 2001, 2002; Raymond et al., 2004; Hains et al., 2004). Specifically, $Na_v1.3$ mRNA was shown to increase after axotomy, as detected by *in situ* hybridization and semi-quantitative RT-PCR (Waxman et al., 1994; Dib-Hajj et al., 1996); to increase by ~twofold between 3 and 7 days after spinal nerve ligation (SNL; Chung model), as determined by RNase protection assay (Kim et al., 2001); and to increase 1.8-fold 10 days after chronic constriction injury (CCI), as determined by quantitative RT-PCR (Hains et al., 2004). Here we report that following peripheral axotomy in the mouse, there was a smaller but still significant increase of 33% in total $Na_v1.3$ mRNA (i.e. isoforms both including and excluding exons 17A+B) whereas expression of the alternatively spliced $Na_v1.3$ mRNA containing exons 17A+B did not change significantly (Fig. 4). This implies that the upregulated $Na_v1.3$ mRNA encodes the functional, four-domain channel, and the significant difference between the expression of $Na_v1.3$ mRNA isoforms after axotomy provides initial evidence for the differential regulation of these isoforms.

The exon 17A sequences of mouse/rat $Na_v1.2$ and rat $Na_v1.3$ mRNAs each include an in-frame TGA termination codon (Fig. 2). In a small number of cases, TGA can specify a selenocysteine (Sec) codon (Okamura et al., 2006) which requires a *cis*-acting stem-loop structure in the 3′ untranslated region of eukaryotes, but no such Sec insertion sequence (SECIS) (Kryukov et al., 2003) was detected in either the published rat $Na_v1.2$ or $Na_v1.3$ cDNA sequences. Therefore, the novel mRNA isoforms of mouse and rat $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ each contain premature termination codons (PTCs), and encode predicted two-domain proteins. Transcripts with PTCs are likely substrates for selective degradation by nonsense-mediated mRNA decay (Amrani et al., 2006). However, the coupling of alternative splicing to the inclusion of an in-frame PTC has been proposed to play a functional role in regulating protein expression (Lewis et al., 2003; Neu-Yilik et al., 2004; Dreumont et al., 2005), and such truncated protein products have been detected in various human tissues (Holbrook et al., 2004; Dreumont et al., 2005; Stojic et al., 2007). In addition to a heterogenous population of neurons, DRG contain Schwann cells and satellite glial cells (Li, 1998; Hanani, 2005), with Schwann cells also having been reported to express $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ (Schaller et al., 1995; Baker, 2002). Therefore it is possible that

expression of predicted two-domain protein isoforms in a subpopulation of cells in the DRG could play a dominant-negative role, especially as they would each still include the ankyrin-G -binding motif VPIAxxESD (Lemaillet et al., 2003; Mohler et al., 2004), but further work will be required to investigate the physiological role of any such proteins.

A predicted two-domain protein is also encoded by the $Na_v1.6$ mRNA isoform containing exon 18N (see introduction), which was proposed as a 'fail-safe' mechanism to prevent the synthesis or activity of full-length protein (Plummer et al., 1997). The mammalian $Na_v1.6$ (*Scn8a*) exon 18N and 18A sequences are conserved in the homologous gene of pufferfish (*Fugu rubripes*)(Plummer et al., 1997), and in both the voltage-gated sodium channel genes *scn8ab* of zebrafish (*Danio rerio*) and $BgNa_V$ (formerly $para^{CSMA}$) of German cockroach (*Blattella germanica*) there is regulated alternative splicing at this site resulting in mRNA isoforms with a PTC that encode predicted two-domain proteins, strongly suggesting a conserved biological function (Tan et al., 2002; Novak et al., 2006b). Among mammalian voltage-gated calcium channel α_1 subunits, which are members of the same super-family and that usually have the same four-domain structure, a predicted two-domain $Ca_v2.2$ protein results from the introduction of a cassette exon with a PTC that is conserved in human, rat and mouse (Raghib et al., 2001). Also, a predicted two-domain $Ca_v1.2$ results from the introduction of a 12 nt segment containing a PTC (Soldatov, 1994; Ertel et al., 2000; Tang et al., 2004) and a 95 kDa, two-domain isoform of $Ca_v2.1$ has been purified that can still interact with its auxiliary β subunit (Scott et al., 1998; Ertel et al., 2000). Heterologously expressed two-domain channels did not produce detectable inward Ca^{2+} currents (i.e. no evidence of homodimerization to form a functional channel), although subcellular localization was similar to four-domain channels (Raghib et al., 2001; Ahern et al., 2001), and co-expression of two-domain and four-domain calcium channels resulted in a dominant-negative suppression of Ca^{2+} currents that may be due to the sequestration of interacting proteins (Raghib et al., 2001; Jeng et al., 2006; Raike et al., 2007).

Human putative exon sequences were identified that have high sequence similarity to the alternatively spliced mouse/rat *Scn2a* and *Scn3a* exons 17A (85-89% identities; Fig. 6), similar to the overall mouse-human exon sequence identity of 87% (Modrek and Lee, 2003). The lower level of conservation of the human putative *SCN9A* ($Na_v1.7$) exon 16A (77% identity) may reflect the lower level of conservation of the surrounding cytoplasmic loop coding region of $Na_v1.7$ (81%, 533/657 nt with single triplet gap; NM_002977 vs. NM_133289) compared with e.g. $Na_v1.2$ (87%, 578/666 nt with no gaps; M94055 vs. NM_012647). To date, no human cDNA sequences containing these sequences have been deposited in public databases, but the full extent of human alternative splicing is far from being fully characterized (Forrest et al., 2006; Stojic et al., 2007). The mouse-human conservation extends into the sequences flanking the alternatively spliced exon, plus internal blocks within the downstream intron (data not shown), consistent with reports of intronic regions flanking alternative exons having significantly higher conservation than those flanking constitutive exons (Sorek and Ast, 2003; Sorek et al., 2004). The *Scn1a/Scn2a/Scn3a/Scn9a* gene cluster on chromosome two in both mice and humans is thought to be the result of tandem duplications that occurred after the split between tetrapods and teleost fish (Plummer and Meisler, 1999; Lopreato et al., 2001; Novak et al., 2006a), but no sequences similar to the alternatively spliced exons 17A of *Scn2a* and *Scn3a* or to exon 16A of *Scn9a* were detected in the mouse, rat or human *Scn1a* ($Na_v1.1$) genes.

The comparison of mammalian and phylogenetically distant chicken sequences has been used recently to address the evolutionary conservation of alternatively spliced products (Katyal et al., 2007; Tang et al., 2007). Here, sequences with 72-76% nucleotide identity to the mouse alternatively spliced exons were identified within the chicken *SCN2A*/locus395945, *SCN3A*/locus424180 and *SCN9A* genes (Fig. 6), but not in the other member

of the chromosome seven gene cluster of sodium channels, the recently identified chicken orthologue of mammalian *SCN1A*, locus 771555 (Table 2 of (Martin et al., 2007); NW_001471729). The gene we refer to as *SCN9A* is still designated as 'locus *SCN1A*' in the current chicken genome build (2.1), although it is the 'putative homologue of mammalian *SCN9A*' and encodes a predicted protein sequence 'similar to PN1' (i.e. *SCN9A*/$Na_v1.7$, (Goldin et al., 2000)) (HomoloGene; XP_422021), and both gene order and orientation are conserved between human/mouse/rat and chicken (http://ncbi.nlm.nih.gov/genome/seq/ (Martin et al., 2007)). Therefore, the alternatively spliced *Scn2a/Scn3a/Scn9a* exon sequences of mouse and rat are conserved in chicken, the divergence time of birds and mammals being ~310 million years ago (International Chicken Genome Sequencing Consortium, 2004).

The identification of these alternatively spliced exon sequences, which are widely conserved, could be of relevance in human disease states. It is known that mutations can affect splice sites of sodium channels (Moric et al., 2003; Mulley et al., 2005; Rossenbacker et al., 2005; Goldberg et al., 2007; Harkin et al., 2007) and that an intronic polymorphism can alter the ratios of alternatively spliced transcripts containing either exons 5N or 5A (Heinzen et al., 2007). The misregulated alternative splicing of *SCN9A* ($Na_v1.7$) exon 16A or of *SCN2A* and *SCN3A* exons 17A could result in either an upregulation of non-functional, predicted two-domain proteins with a concomitant downregulation of functional, four-domain channels, or vice versa. Gain-of-function mutations of *SCN9A* cause primary erythermalgia (Yang et al., 2004; Cummins et al., 2004) and paroxysmal extreme pain disorder (Fertleman et al., 2006), whereas loss-of-function mutations are associated with the inability to sense pain (Cox et al., 2006; Goldberg et al., 2007), and *SCN2A* mutations have been detected in patients with epilepsy (Striano et al., 2006; Scalmani et al., 2006; Herlenius et al., 2007) and in a case of autism (Weiss et al., 2003). Therefore, screening of these putative exons and their flanking sequences for mutations could be informative in various human diseases associated with these channels. It is also possible that species-specific control of *SCN9A* ($Na_v1.7$) alternative splicing could help account for the difference in phenotype between the human loss of pain sensation due to loss-of-function mutations (Cox et al., 2006; Goldberg et al., 2007) and the perinatal lethality of $Scn9a^{-/-}$ knockout mice (Nassar et al., 2004).

CONCLUSION

In summary, we describe the isolation of alternatively spliced mRNA isoforms of mouse and rat $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$, each with novel exons that include an in-frame stop codon and therefore encode predicted two-domain, truncated proteins. Currently, it is not known whether the expression of these alternatively spliced mRNAs acts as a 'fail-safe' mechanism to prevent four-domain channel synthesis via making a substrate for nonsense-mediated mRNA decay, or is for the synthesis of two-domain proteins that may still interact with ankyrin-G. Whichever proves to be the case, we propose that the widespread conservation of these sequences (rodents/human/bird) strongly suggests an additional level of regulation in these voltage-gated sodium channels.

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Abbreviations

bp	base pairs
DRG	dorsal root ganglia
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
kb	kilobases
nt	nucleotides
PTC	premature termination codon
RT	reverse transcription
TTX	tetrodotoxin

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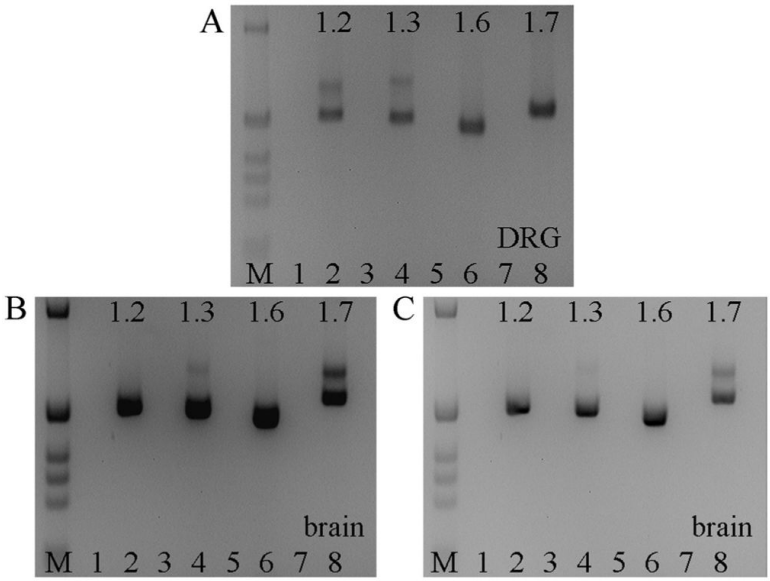

Fig. 1.

The expression of $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ mRNAs in adult mouse DRG and brain. (A) RT-PCR analysis of sodium channel expression in adult mouse DRG showing larger, minor isoforms of $Na_v1.2$ and $Na_v1.3$. Products of the expected sizes for $Na_v1.2$ (lane 2), $Na_v1.3$ (lane 4), $Na_v1.6$ (lane 6) and $Na_v1.7$ (lane 8) were amplified from reverse-transcribed RNA, whereas no products were detected in the corresponding RT-minus controls (respectively, lanes 1, 3, 5 and 7). (B) RT-PCR analysis of sodium channel expression in adult mouse brain, showing larger, minor isoforms of $Na_v1.3$ and $Na_v1.7$ (shorter exposure shown in C). Lane designations in (B) and (C) are as in (A). M is 1 kb DNA ladder (Invitrogen) showing fragments of 1018, 517/506, 396, 344, 298 and 220/201 bp. The expected RT-PCR product sizes were 541 bp ($Na_v1.2$), 534 bp ($Na_v1.3$), 515 bp ($Na_v1.6$) and 575 bp ($Na_v1.7$).

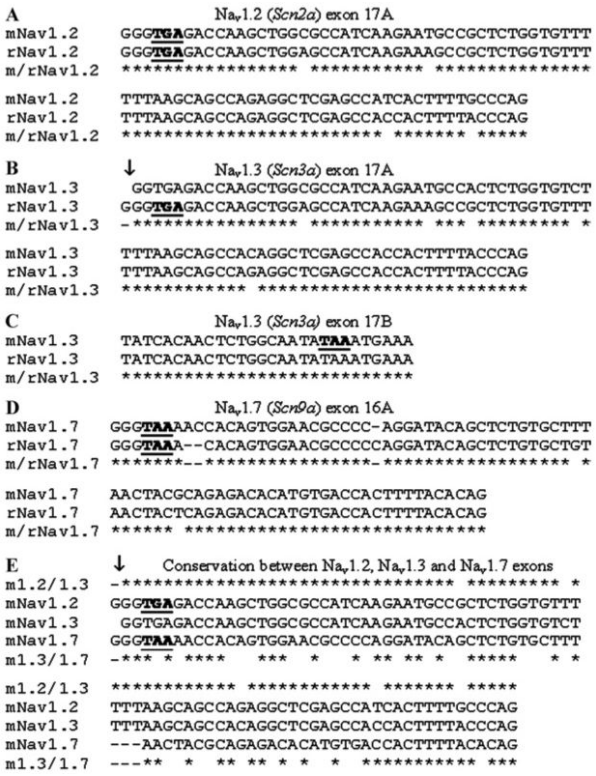

Fig. 2.

The alternatively spliced exon sequences of mouse and rat Na$_v$1.2, Na$_v$1.3 and Na$_v$1.7 mRNAs, showing in-frame termination codons (underlined bold). (A) Alignment of Na$_v$1.2 (*Scn2a*) exon 17A sequences of mouse (m) and rat (r), with conserved nt (m/r) indicated below by asterisks. (B) Na$_v$1.3 (*Scn3a*) exon 17A sequences; (C) Na$_v$1.3 (*Scn3a*) exon 17B sequences; and (D) Na$_v$1.7 (*Scn9a*) exon 16A sequences. (E) Alignments of the mouse Na$_v$1.2, Na$_v$1.3 and Na$_v$1.7 alternatively spliced exon sequences, with conserved nt between Na$_v$1.2 and Na$_v$1.3 (m1.2/1.3) and between Na$_v$1.3 and Na$_v$1.7 (m1.3/1.7) shown, respectively, above and below by asterisks. The downward arrow in (B) and (E) indicates the site of the relative deletion of a single nucleotide in mouse Na$_v$1.3 exon 17A that results in the reading frame extending downstream into exon 17B, and '-' indicates a gap introduced to optimize an alignment.

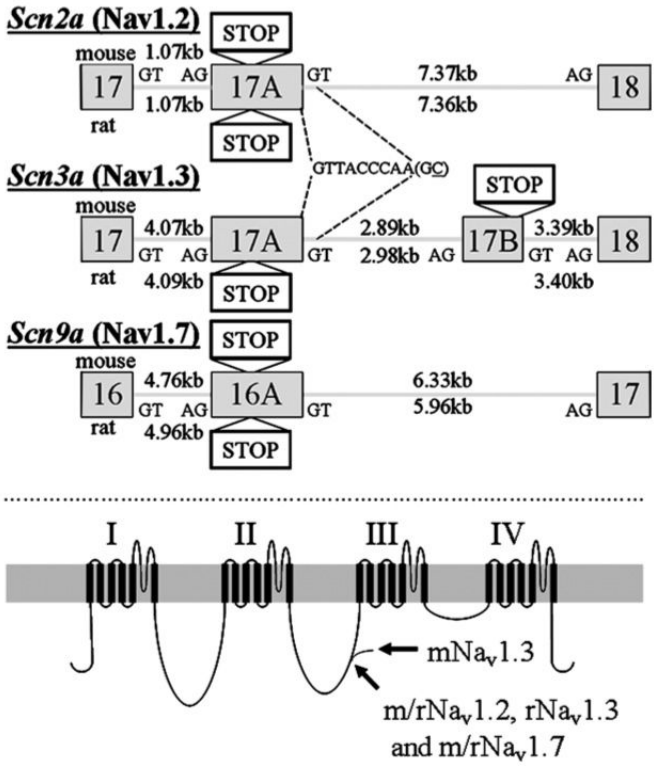

Fig. 3.

Schematic diagram of the mouse and rat *Scn2a* ($Na_v1.2$), *Scn3a* ($Na_v1.3$) and *Scn9a* ($Na_v1.7$) genes in the regions of the novel alternatively spliced exons. Exons are represented by boxes and introns by lines (not to scale), with constitutive exons numbered as previously published (Kasai et al., 2001; Yang et al., 2004; Cox et al., 2006) and in-frame stop codons within the alternatively spliced exons of mouse and rat indicated above and below, respectively. Intron lengths of the mouse and rat genes are in kb, and canonical GT-AG dinucleotides present at the donor and acceptor splice sites in both mouse and rat are shown. The downstream donor splice site selections at exons 17A that can result in nine nucleotide extensions of the rat $Na_v1.2$ and mouse $Na_v1.3$ mRNAs are indicated by dotted lines. Shown below is the predicted topology of a generic voltage-gated sodium channel α-subunit within the plasma membrane (gray), with arrows indicating the corresponding predicted termination sites (X, stop codons) within the second cytoplasmic loop that result from alternative splicing of mouse and rat (m/r) $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ mRNAs. The alternatively spliced mouse $Na_v1.3$ mRNA isoforms encode either 34 or 37 novel C-terminal amino acids.

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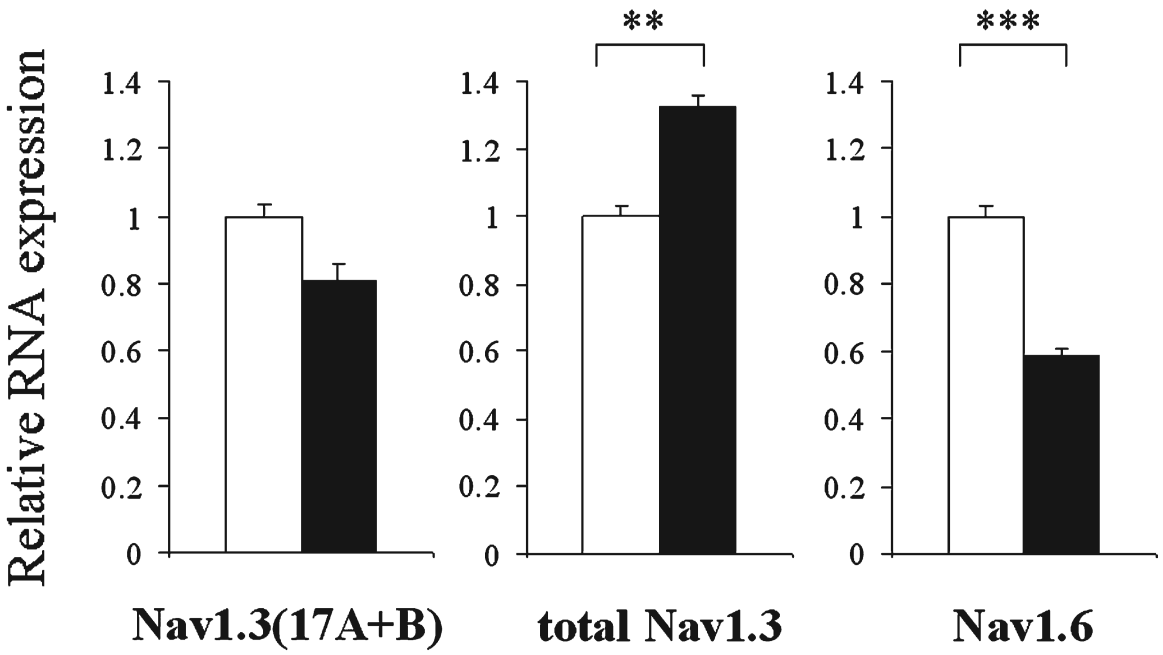

Fig. 4.

The expression of $Na_v1.3$ mRNA isoforms in adult mouse DRG 1 week after axotomy. In quantitative RT-PCR assays the expression of $Na_v1.3$ mRNA containing exons 17A and 17B (17A+B) was unchanged (n=5; 0.810±0.049 of control, not significant with P>0.05), total $Na_v1.3$ mRNA increased to 1.328±0.030 of control (n=5; P=0.003) and $Na_v1.6$ mRNA decreased to 0.586±0.025 of control (n=5; P<0.001) in pooled ipsilateral (axotomized) lumbar L4 and L5 DRG compared with contralateral (unaxotomized) controls 1 week after axotomy. Data are shown as means±S.E., in which expression after axotomy (filled boxes) was compared with contralateral controls of 1.00 relative units (unfilled boxes). Two asterisks, P<0.005; three asterisks, P<0.001.

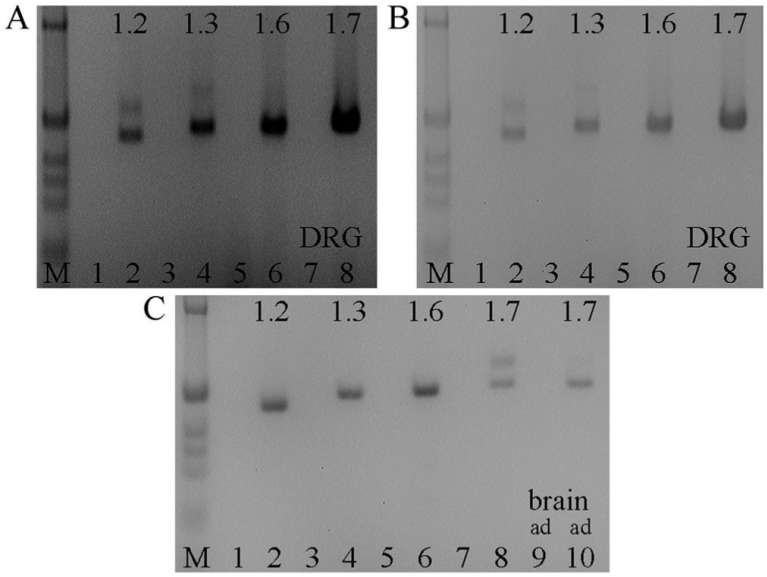

Fig. 5.

The expression of $Na_V1.2$, $Na_V1.3$, $Na_V1.6$ and $Na_V1.7$ mRNAs in adult rat DRG and neonatal brain. (A) RT-PCR analysis of sodium channel expression in adult rat DRG showing larger, minor isoforms of $Na_V1.2$ and $Na_V1.3$ (shorter exposure shown in B). (C) RT-PCR analysis of sodium channel expression in neonatal rat brain showing a larger isoform of $Na_V1.7$ (lane 8), not detected in adult rat brain (lane 10). Lane designations and DNA ladder fragment sizes are as in Fig. 1, except for (C) adult (ad) brain lanes 9 (RT-minus, $Na_V1.7$) and 10 (RT-plus, $Na_V1.7$). An erratic band of ~300 bp detectable in some rat samples amplified with $Na_V1.6$ primers is a misprimed product unrelated to sodium channels (data not shown). The expected RT-PCR product sizes were 474 bp ($Na_V1.2$), 505 bp ($Na_V1.3$), 519 bp ($Na_V1.6$) and 537 bp ($Na_V1.7$).

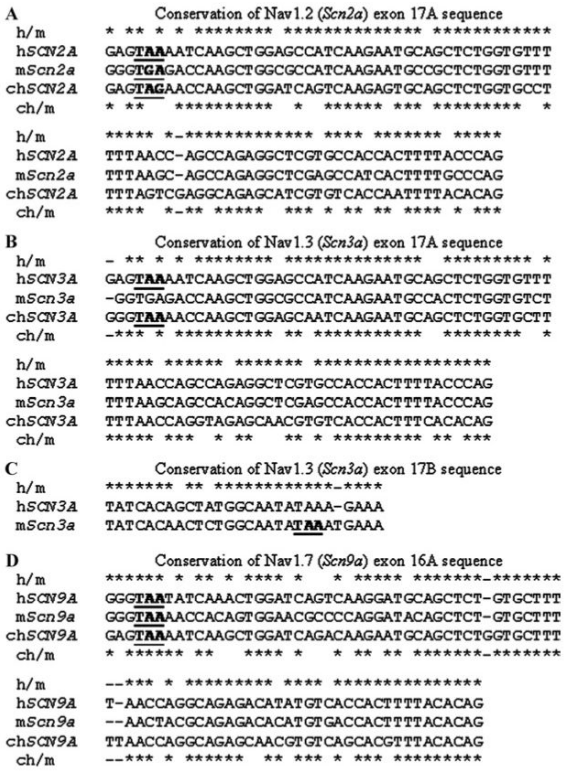

Fig. 6.

The human and chicken putative exon sequences with high levels of conservation to the alternatively spliced exons of mouse and rat $Na_V1.2$ (*Scn2a*), $Na_V1.3$ (*Scn3a*) and $Na_V1.7$ (*Scn9a*) mRNAs. The sequences of the human (h) and chicken (ch) putative exon 17A of *SCN2A* (A), exon 17A of *SCN3A* (B), exon 17B of *SCN3A* (C) and exon 16A of *SCN9A* (D) are each shown with conserved nt between human and mouse (h/m) and between chicken and mouse (ch/m) indicated, respectively, above and below by asterisks. Note that no sequence homologous to *SCN3A* exon 17B was detected in chicken. Chicken gene designations for *SCN2A* follow locus 395945 and published work (Striano et al., 2006; Martin et al., 2007); *SCN3A* follows Martin et al. (2007) and is currently listed as locus 424180 in genome build 2.1; and for *SCN9A*, see Discussion. Underlined bold capitals are putative in-frame termination codons, and '-' indicates a gap introduced to optimize an alignment.

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Table 1

Intron-exon boundaries of the alternatively spliced *Scn2a* (Nav1.2), *Scn3a* (Nav1.3) and *Scn9a* (Nav1.7) exons

Gene	Acceptor site	Exon	Donor site
m*Scn2a*	cttctcacag**GGGTGA**	17A	**GCCCAG**gttacccaagcaagt
r*SCN2A*	cttctcacag**GGGTGA** **********	17A	**ACCCAGgttacccaa**gcaagt **********
			↑
			↓
m*Scn3a*	cttctcacag**GGTGAG**	17A	**ACCCAGgttacccaa**gcaagt
r*SCN3A*	cttctcacag**GGGTGA** **********	17A	**ACCCAG**gttacccatgcaagt ******** ******
m*Scn3a*	atcattgtag**TATCAC**	17B	**ATGAAA**gtaagttttt---cc
r*SCN3A*	atcattgtag**TATCAC** **********	17B	**ATGAAA**gtaagttttttttcc **********---**
m*Scn9a*	acctctgcag**GGGTAA**	16A	**ACACAG**gttacctaaaacatc
r*SCN9A*	acctctgcag**GGGTAA** **********	16A	**ACACAG**gttacctaaaatatc ********** ***

Mouse (m) and rat (r) splice acceptor and donor site sequences flanking the alternatively spliced exons (bold capitals) are shown, with conserved intron nucleotides indicated below by asterisks and '-' indicating a gap introduced to optimize an alignment. Underlined exon sequences are in-frame termination codons, and arrows indicate the variant downstream donor splice sites of rat SCN2A and mouse Scn3a exons 17A that result in the inclusion of nine additional nucleotides (bold, lowercase).