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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.

Key words: 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 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.

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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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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′