Trans-splicing of a voltage-gated sodium channel is regulated by nerve growth factor

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Abstract Mammalian sensory neurons express a voltage-gated sodium channel named SNS. Here we report the identification of an SNS transcript (SNS-A) that contains an exact repeat of exons 12, 13 and 14 encoding a partial repeat of domain II. Because the exons 12–14 are present in single copies in genomic DNA, the SNS-A transcript must arise by trans-splicing. Nerve growth factor, which regulates pain thresholds, and the functional expression of voltage-gated sodium channels increases the levels of the SNS-A transcript several-fold both in vivo and in vitro as measured by RNase protection methods, as well as RT-PCR. These data demonstrate a novel regulatory role for the nerve growth factor and are the first example of trans-splicing in the vertebrate nervous system.

Key words: Trans-splicing nerve growth factor; Sensory neuron specific; dorsal root ganglia

1. Introduction

Voltage-gated sodium channels play an essential role in the generation and propagation of action potentials [1]. In adult rat dorsal root ganglia (DRG) neurons in culture, large diameter cells express tetrodotoxin-sensitive (TTXs) sodium currents, whilst small neurons also express tetrodotoxin-insensitive (TTXi) currents [2–5]. A TTXi sodium channel α-subunit named sensory neuron specific (SNS) accounts for the TTXi sodium currents described in small diameter sensory neurons [6,7].

Transcriptional regulation of sodium channel expression by nerve growth factor (NGF) has been demonstrated [8]; NGF application has been shown to cause dramatic decreases in thermal, chemical and mechanical thresholds of pain perception in animal models [9]. It seemed plausible that transcriptional regulation of sodium channel expression by NGF might play a role in the development of NGF-mediated hyperalgesia. We therefore examined the levels of the SNS α-subunit in inflammatory models and in NGF-treated neurons in culture. We found a small effect on the total amount of SNS mRNA consistent with earlier studies [10]. In contrast, there was a dramatic effect of NGF on a splice variant of SNS both in vitro and in vivo. Sequence analysis showed that the variant, named SNS-A, contains an identical repeat of three exons, encoding four transmembrane regions of domain II, including a partial S4 domain that comprises a voltage sensor. The splice variant is induced several-fold by NGF. Because no exon duplications are present between exons 11 and 15 in either mouse or rat genomes, the SNS variant must arise by a form of trans-splicing, a phenomenon never previously observed in a vertebrate structural gene.

2. Materials and methods

Southern blots, Northern blots and DNA sequencing were carried out as previously described in detail [11].

3. Detection and quantitation of SNS and SNS-A

Adult rat DRG neurons were cultured in the presence of NGF (50 ng/ml) and cytosine-β-D-arabinofuranoside (10 μM) for the first 3 days. Cultures were then supplemented with NGF (50 ng/ml) or grown in the absence of NGF and presence of rabbit anti-NGF antiserum (Sigma) for 7 days. Alternatively, 2 day old rats were treated with 2 μg NGF i.p. 24 h before DRG removal. Total RNA was extracted from the culture or whole DRG. SNS-A was detected by RT-PCR and quantitated with RNase protection assays. 5 μg total RNA from the DRG neurons cultured with or without NGF were treated with DNase I and cDNA was synthesised with Superscript reverse transcriptase using random hexamer primers in a total volume of 20 μl. 1 μl of the reverse transcribed solution was used for PCR (32 cycles: 94°C, 1 min: 55°C, 1 min: 72°C, 1 min) in the presence of the primer pairs specific for splice variant SNS, total SNS and CGRP. Primer sequences used in the PCR are as follows: SNS-A (393 bp): 5′-CAGCTTCGCTCAGAAGTA-3′, 5′-TGCCCTGTAGTGTGCAGT-3′: total SNS (168 bp) 5′-AGCACGGTGGACTGCCCGGA-3′, 5′-GCGCACCTGCCAGCCTGT-3′: CGRP (223 bp), 5′-GGTGTGGTGAAGGACAAC-3′, 5′-TCATAGGGAGAAGGG-3′.

L-27 primers were added to the PCR reaction six cycles after the start. Each PCR experiment was carried out at least four times and gave identical results. RNase protection assays were performed as previously described [10]. Briefly, 5 μg total RNA from the DRG neurons cultured with or without NGF was hybridised with ^{32}P-labelled cRNA probe specific for total SNS, splice variant SNS, cyclophilin, and CGRP in the presence of 80% formamide at 45°C for 12 h. Each hybrid-

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Abbreviations: DRG, dorsal root ganglia; NGF, nerve growth factor; SNS, sensory neuron specific; TTXi, tetrodotoxin-insensitive; TTXs, tetrodotoxin-sensitive

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isation reaction was treated with 20 μg/ml RNase A and 1 μg/ml RNase T1 at 33°C for 30 min. The reaction was stopped by proteinase K, extracted with phenol/chloroform, precipitated with ethanol and redissolved in 10 μl RNA loading buffer. The protected bands were analysed on a denaturing polyacrylamide/urea gel. Scanned images were quantitated using the public domain NIH image programme written by W. Rasband and available from the internet by anonymous ftp from zippy.nimh.nih.gov. The 359 bp probe for total SNS mRNA was derived from an *Sph*I fragment in the 3′ untranslated region of SNS and SNS-A. A probe that distinguished SNS (373 bp) from the SNS-A variant of 393 bp was generated from the PCR product generated using primers 24 and 26 (see Fig. 2). The CGRP probe of 223 bp comprised part of CGRP exons 5 and 6. The cyclophilin probe comprises 300 bp of the coding sequence.

4. Results

4.1. Identification of an SNS splice variant

Sequencing of cDNA clones from a Sprague Dawley oligo dT- primed cDNA library encoding SNS sodium channels led to the identification of a novel α-subunit transcript named SNS-A (one in six of the full length positive clones isolated) that contained a 525 bp insert at position 1755 in interdomain region 1 (ID1). Sequencing of this insert demonstrated that it comprised an exact repeat of part of ID1 and the S1–3 and part of the S4 domains present in domain 2, whilst the re-

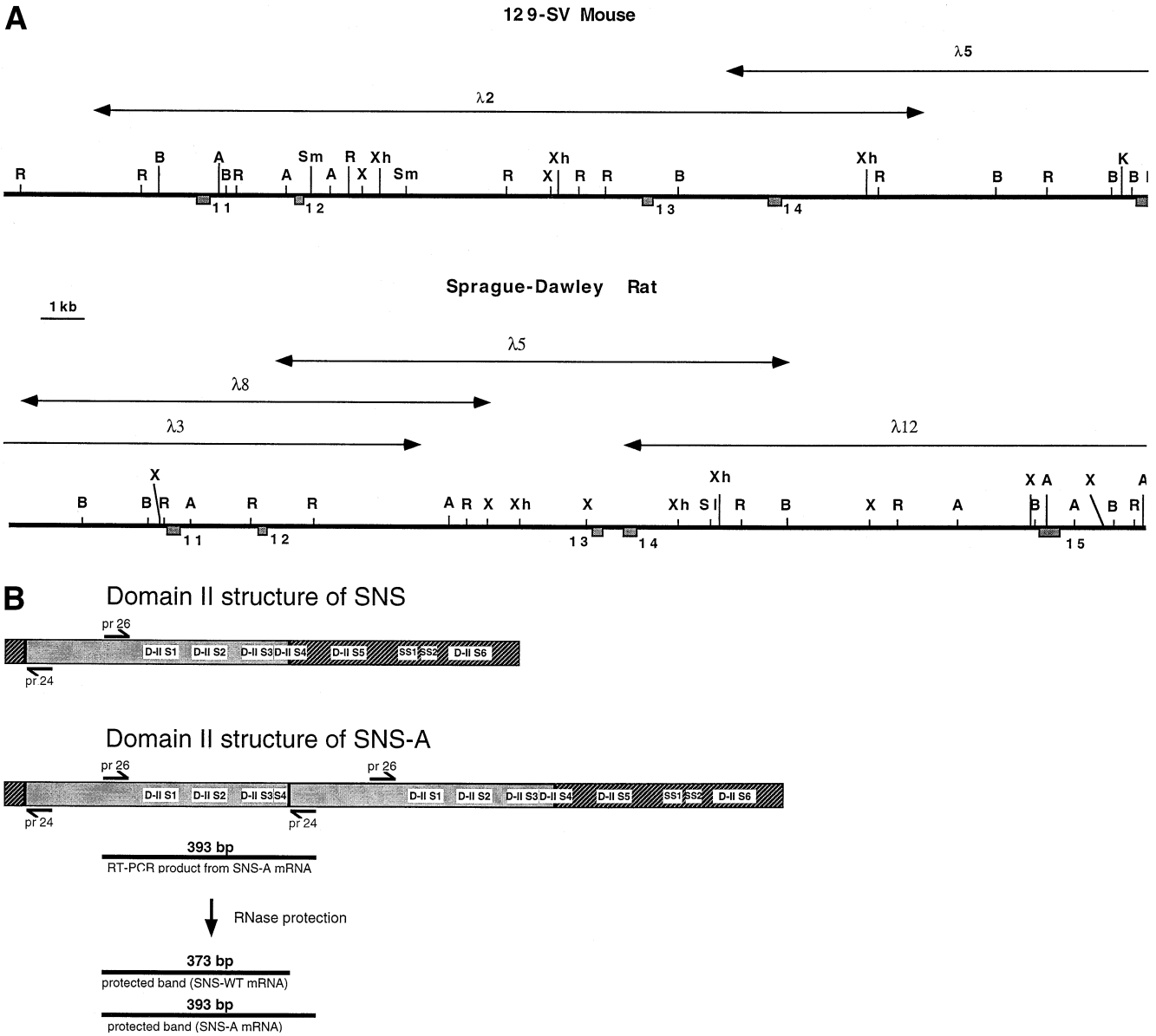

Fig. 1. A: genomic structure of SNS domain II. A restriction map of the genomic locus containing exons 11–15 of the *sns* gene in either mouse 129/SV or Sprague-Dawley rat genomic libraries is shown. The region was mapped with *Apa*I (A), *Bam*HI (B), *Kpn*I (K), *Eco*RI (R), *Sal*I (SI), *Sma*I (Sm), *Xba*I (X) and *Xho*I (Xh) restriction enzymes and analysed by cross hybridisation studies. B: Structure of SNS and SNS-A and location of PCR primers. A schematic drawing of the location of the repeat present in SNS-A (a 525 bp insert at position 1755 in ID1) and its relation to the normal domain structure of SNS is shown. The location of the PCR primers shown in the Section 2 used to identify the splice variant and to generate an RNase protection probe which will distinguish normal and spliced transcripts is shown.

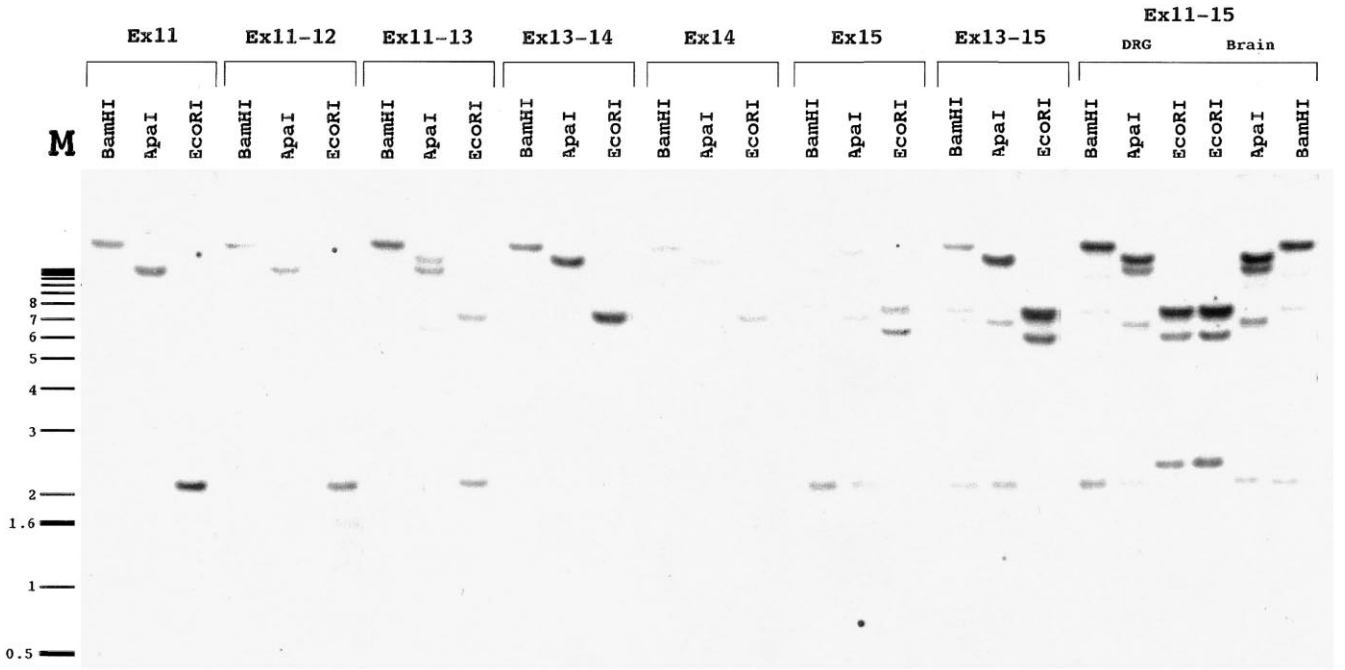

Fig. 2. Southern blots of rat DNA from DRG or brain show that exons 12–14 are not duplicated. Using three restriction enzymes and exon specific probes (see Figure 1), genomic DNA digests from DRG and brain (right panel) were analysed by Southern blotting. A 1 kb ladder is marked on the left of the figure. The sizes of restricted bands are:

	Exon 11	Exon 12	Exon 13	Exon 14	Exon 15
*Eco*RI	2.1	1.5	6.8	6.8	5.7
*Bam*HI	15	15	15	15	2
*Apa*I	11	6.1	12.5	12.5	0.7 and 2.0

demonstrating the presence of single copies of the probed exons.

mainder of the clone including the 5′ and 3′ UTRs corresponded exactly to the sequence of the native transcript (Fig. 1). The exon-intron structure and splice donor and acceptor sites for *sns* have previously been described [11] and the insert corresponds precisely to a repeat of exons 12, 13 and 14. We therefore examined the genomic structure of the *sns* gene in mouse and rat, in order to detect the existence of any repeated exons. Using a combination of sequencing and Southern blotting with overlapping clones that are shown in Fig. 1, we carefully analysed the genomic organisation of exons 12, 13 and 14 that encode the apparent sequence repeat [11].

In Fig. 1A, a map of the locus of the rat genomic *sns* clone is shown. Genomic rat DNA in λ-fix II was digested with *Eco*RI and either *Eco*RI with *Sal*GI, or *Eco*RI and *Not*I. The (*Not*I and *Sal*GI sites are adjacent to each other in the lambda fix II polylinker). The digests were Southern-blotted and hybridised with cDNA fragments of exon-specific probes. A single *Eco*RI 2.1 kb digestion product hybridised with the exon 11 specific clone. A 1.5 kb *Eco*RI fragment hybridised with probes for exons 11, 12 and 13, but not exons 11 or exons 13 and 14. This 1.5 kb fragment must therefore contain exon 12 alone. Hybridisation of the λ5 and λ12 clones with probes for exons 13 and 14, exons 11, 12 and 13 and an exon 11 specific probe shows that the 6.8 kb fragment contains exons 13 and 14. λ12 contains exon 14 but not exons 11, 12 and 13. There is no exon duplication for 6.8 kb downstream of exon 14. Exon 15 was localised by hybridisation with probes for exons 11–15. A 5.7 kb *Eco*RI fragment of λ12 contains only exon 15. Taken together these data (not shown) demonstrate that there is no duplication of exons 12–14 between exons 11 and 15. Similar data were obtained using mouse 129/sv genomic DNA.

We analysed Sprague-dawley rat genomic DNA by Southern blotting to determine whether exons 12–14 might exist in the genome outside the locus covered by the genomic clones. To discount any possible rearrangement of DNA in DRG, we analysed DNA extracted from both DRG and brain, restricted with one of the three enzymes (Fig. 2).

The data presented in Fig. 2 confirm the fact that exons 12–14 encoding the putative repeat are only present in single copies in the genome. The possibility that the SNS-A transcript could be a cloning artefact was tested by examining the occurrence of SNS-A like transcripts in RNA extracted from DRG. We devised both a PCR strategy and an RNase protection protocol to examine the occurrence of the splice variant transcript in both cDNA and RNA derived from DRG sensory neurons (Fig. 3).

Fig. 1B demonstrates the primers that were used to distinguish between the wild-type SNS and variant SNS-A channels. Because of their orientations, primers 24 (CTGCCCTGTAGTGTGAGT) and 26 (CAGCTTCGCTCAGAAGTA) will only produce a PCR product of a size of 393 bp with the splice variant, but not with the wild-type transcript. We sequenced the PCR product to confirm that the sequence corresponds to SNS-A. Using the PCR product from these primers, RNase protection probes were also generated that would give distinct size products of 373 bp for SNS mRNA

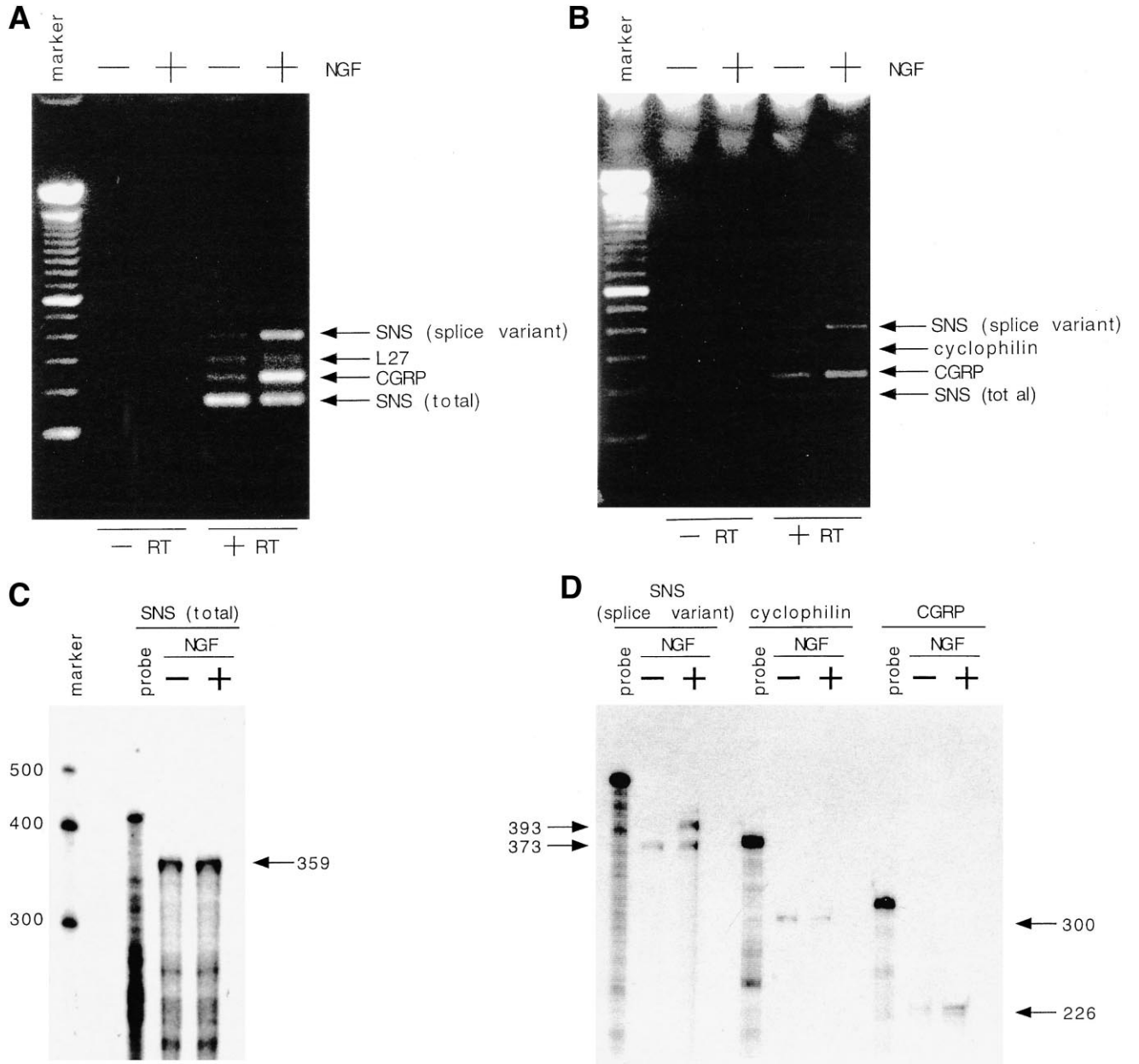

Fig. 3. NGF regulation of SNS-A expression. A: RT-PCR analysis of SNS and SNS-A. RNA extracted from adult rat DRG neurons grown in the absence (−) or presence (+) of NGF for one week was used to generate random primed cDNA that was subjected to RT-PCR as described in 2. In the absence of reverse transcriptase, no PCR products were generated (−RT). In the presence of reverse transcriptase (+RT), levels of the control ribosomal transcript L-27 are similar in + and −NGF samples. The levels of CGRP and the sodium channel splice variant SNS-A are clearly up-regulated, whilst the total amount of SNS transcript is little affected by variations in NGF treatment. B: In vivo studies. Male and female rats were treated with 0.5 μg/g i.p. NGF for 24 h and DRG was removed and RNA extracted from control and experimental litter mates. In the absence of reverse transcriptase, no PCR products were generated (−RT). In the presence of reverse transcriptase (+RT), levels of the control cyclophilin transcript are similar in + and −NGF samples. The levels of CGRP and the sodium channel splice variant SNS-A are clearly up-regulated to similar levels, whilst the total amount of SNS transcript is little affected by NGF treatment in four out of four experiments. C and D: RNase protection analysis of SNS and SNS-A levels in NGF-treated DRG neurons. Using the probe derived from the PCR products, generated using primers 24 and 26 (see Figure 1B), and probes derived from the common 3′ region of SNS and SNS-A, the effect of NGF on the levels of the transcripts was investigated. C: The levels of total SNS transcripts are shown to be slightly up-regulated in the presence of NGF, when normalised to the levels of cyclophilin mRNA. D: The 2 products of RNase protection are shown. The larger 393 bp protected band representing the level of SNS-A transcripts is strongly up-regulated by NGF treatment, as is the mRNA encoding CGRP (D). Cyclophilin levels are little changed and scanned bands are normalised to cyclophilin to provide estimates of the level of NGF induction of the transcripts shown.

and 393 bp for SNS-A mRNA. Using these two approaches, it can be seen (Fig. 3) that the splice variant occurs not only in DRG-derived cDNA but also in RNA extracted from these cells, precluding the possibility that SNS-A represents a cloning artefact.

4.2. NGF regulation of SNS-A expression

The effect of NGF on the levels of expression of SNS and the SNS-A transcript was first examined using adult DRG neurons grown in culture. CGRP was used as a positive control to examine the effect of NGF on transcript levels which

are known to be NGF-regulated. Cyclophilin and L-27 are ubiquitously expressed genes, which are not regulated in expression by NGF and we therefore normalised the levels of protected transcripts in RNase protection experiments to cyclophilin or to the ribosomal protein L-27 transcript to obtain semi-quantitative data on NGF induction [12].

RT-PCR was first used to examine the relative levels of SNS and SNS-A in DRG-derived cDNA. As can be seen in Fig. 3A, there is a dramatic increase in SNS-A levels on NGF treatment. We carried out similar experiments in neonatal rats, where we found a similar level of increase of both splice variant and CGRP transcripts in systemic treatment with NGF (Fig. 3B). In contrast to the in vitro studies, anti-NGF antisera had no effect on baseline levels of CGRP and SNS-A, probably because the time course of the in vivo experiment was only 24 h. In order to quantitate the induction of the splice variant by NGF, we carried out RNase protection experiments. After 7 days in culture, there was a 6-fold alteration in the levels of CGRP mRNA, whilst cyclophilin levels were unaltered. Interestingly, the level of mRNA encoding the splice variant SNS-A increased 8-fold, whilst the total level of mRNA showed little change (15–20%) on NGF addition (Fig. 3C,D). Western blotting has already demonstrated that there is a small increase in immunoreactive SNS on NGF treatment of adult DRG neurons, whilst total mRNA levels are up-regulated to a limited extent (about 20%) [10].

The regulation of sodium channel activity in sensory neurons has been linked to altered pain thresholds [18]. NGF is known to cause peripheral hyperalgesia [15]. NGF has been shown to influence the splice choice in PC12 cells, causing altered expression of both beta-amyloid precursors and agrin splice variants [13,14]. Post-transcriptional regulation of potassium channel expression by NGF has been observed using Western blots combined with RNase protection in PC12 cells [15]. Here, administration of NGF led to a 6-fold increase in protein and channel density, but the level of mRNA transcript was apparently unaffected, although the possibility that alternative splicing occurred was not explored. Alternative splicing of many VGSCs, sometimes leading to channels with distinct functional properties has been described [16].

The mechanism that gives rise to the NGF-induced variant SNS-A is not yet understood. The evidence that the exon 12–14 repeat present in the mRNA transcript is not present in genomic DNA is good, suggesting that some from of trans-splicing must occur. Splicing involves the actions of small nuclear RNAs and over 50 protein components that form spliceosomes that act with remarkable fidelity to excise intronic sequences. Trans-splicing has been observed in yeast [17] in trypanosomes [18] and in *Caenorhabditis elegans* [19]. There is good evidence that mammalian cells are able to trans-splice RNA derived from studies of mRNA from simple eukaryotes, or viral transcripts encoding SV40 T-antigen in nuclear injected mammalian cell lines and nuclear extracts [20,21]. There is also evidence for trans-splicing in the immune system. Germ line transcripts from Ig heavy chain loci precede the occurrence of isotype switching. A reverse transcriptase-PCR approach detected human chimeric Ig germ line mRNA transcripts in human B-cells stimulated with IL-4 [22]. Sequence analysis revealed that all of these chimeric Ig germ line transcripts represented the exons from one Ig locus spliced to the CH exons from another locus using consensus splicing donor and acceptor sites, indicating that they were generated through splicing machinery. Similarly a CAM Kinase II transcript present in islets of Langerhans has been proposed to arise by trans-splicing [23]. The mechanisms involved in *cis*- and *trans*-splicing may be closely related. A family of Serine-Arginine rich RNA binding proteins known as SR proteins have been shown to play a critical role in the formation of active spliceosomes. Interestingly, peripheral nerve crush has been shown to regulate the level of expression of one such protein, Srp20 [24].

The functional significance of NGF-mediated regulation of SNS splice variants in nociception is not yet clear. Attempts to express the SNS-A variant in a functional form in heterologous expression systems have failed. However, the apparent lack of activity of SNS-A does not preclude an effect on neuronal excitability in vivo. Effects of the SNS-A splice variant on channel trafficking, the subcellular localisation of the functional SNS channel or the half life of functional channels, although not apparent in dissociated tissue culture, may regulate nociceptive thresholds in vivo.

In summary, we have demonstrated the existence of a novel splice variant of SNS that contains a three exon repeat in domain II, and encodes a translatable protein. NGF induces the appearance of this splice variant in vitro and in vivo. The only plausible explanation for the appearance of this transcript is trans-splicing. Although evidence for trans-splicing using RT-PCR has been obtained in the mammalian immune system and cell lines, the use of RNase protection in the present study provides compelling evidence for the existence of trans-spliced transcripts. The regulation of this event by NGF identifies yet another locus of regulatory action for the neurotrophins.

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References

[1] Hille, B. (1992) Ionic Channels of Excitable Membranes, 2nd edn., Sinauer, Sunderland, USA.
[2] Roy, M.L. and Narahashi, T. (1992) J. Neurosci. 12, 2104–2111.
[3] Ogata, N. and Tatebayashi, H. (1992) Dev. Brain Res. 65, 93–100.
[4] Elliott, A.A. and Elliott, J.R. (1993) J. Physiol. London 463, 39–56.
[5] Omri, G. and Meiri, H. (1990) J. Membr. Biol. 115, 13–29.
[6] Akopian, A.N., Sivilotti, L. and Wood, J.N. (1996) Nature 379, 257–262.
[7] Sangameswaran, L., Delgado, S.G., Fish, L.M., Koch, B.D., Jakeman, L.B., Stewart, G.R., Sze, P., Hunter, J.C., Eglen, R.M. and Herman, R.C. (1996) J. Biol. Chem. 271, 5953–5956.
[8] Tanaka, M., Cummins, T.R., Ishikawa, K., Dib-Hajj, S.D., Black, J.A. and Waxman, S.G. (1998) Neuroreport 20, 967–972.
[9] Lewin, G.R., Rueff, A. and Mendell, L.M. (1994) Eur. J. Neurosci. 6, 1903–1912.
[10] Okuse, K., Chaplan, S.R., McMahon, S., Luo, Z.D., Calcutt, N.A., Scott, B.P., Akopian, A.N. and Wood, J.N. (1997) Mol. Cell. Neurosci. 10, 196–207.
[11] Souslova, V.A., Fox, M., Wood, J.N. and Akopian, A.N., Genomics 41, pp. 201–209.
[12] Winter, J., Forbes, C.A., Sternberg, J. and Lindsay, R.M. (1988) Neuron 1, 973–981.
[13] Smith, M.A., Fanger, G.R., O'Connor, L.T., Bridle, P. and Maue, R.A. (1997) J. Biol. Chem. 272, 15675–15681.
[14] Sharma, N., D'Arcengelo, G., Kleinklaus, A., Halegoua, S. and Trimmer, J.S. (1996) J. Cell Biol. 123, 1835–1843.

[15] Auld, V.J., Goldib, A.L., Krafte, D.S., Marshall, J., Dunn, J.M., Catterall, W.A., Lester, H.A., Davidson, N. and Dunn, R.J. (1988) Neuron 1, 449–461.
[16] Ghetti, A. and Abelson, J.N. (1995) Proc. Natl. Acad. Sci. USA 92, 11461–11464.
[17] Lucke, S., Klockner, T., Palfi, Z., Boshart, M. and Bindereif, A. (1997) EMBO J. 16, 4433–4440.
[18] Maroney, P.A., Denker, J.A., Darzynkiewicz, E., Laneve, R. and Nilsen, T.W. (1995) RNA 1, 714–723.
[19] Eul, J., Graessmann, M. and Graessmann, A. (1995) EMBO J. 14, 3226–3235.
[20] Eul, J., Graessmann, M. and Graessmann, A. (1996) FEBS Lett. 394, 227–232.
[21] Fujieda, S., Lin, Y.Q., Saxon, A. and Zhang, K. (1996) J. Immunol. 157, 3450–3459.
[22] Breen, M.A. and Ashcroft, S.J. (1997) FEBS Lett. 409, 375–379.
[23] Gillen, C., Gleichmann, M., Spreyer, P. and Muller, H.W. (1995) J. Neurosci. Res. 42, 159–171.
[24] Yang, N., George, A.L. and Horn, R. (1996) Neuron 16, 113–122.