The Role of Tetrodotoxin-resistant Sodium Channels in Pain Sensation

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Abstract

Nociceptive dorsal root ganglion neurons express sensory neuron-specific tetrodotoxin-resistant voltage-gated sodium channel (SNS). Here, we examined the role of SNS in nociception by constructing sns-knockout mice. The sns-knockout mice expressed only TTX-sensitive sodium currents on step depolarizations from normal resting potentials, demonstrating that all slow TTX-resistant currents are mediated by the sns gene. The mutant mice were viable, fertile and apparently normal, although lowered thresholds of electrical activation of C-fibers and increased current densities of TTX-sensitive sodium channels demonstrated compensatory up-regulation of TTX-sensitive currents in DRG neurons. Behavioral studies demonstrated a pronounced analgesia to noxious mechanical stimuli, small deficits in noxious thermoreception and delayed development of inflammatory hyperalgesia. These data show that SNS is involved in pain sensation and suggest that blockade of SNS expression or function may produce notable analgesia without significant side effects.

Key words: sodium channel, pain sensation, tetrodotoxin, dorsal root ganglion

INTRODUCTION

Several distinct types of voltage-gated sodium channels are expressed in dorsal root ganglion (DRG) neurons. The current mediated by the sensory-neuron-specific TTX-resistant sodium channel (SNS) is insensitive to micromolar concentrations of tetrodotoxin (TTX), with a low single-channel conductance, slow activation and inactivation kinetics and a more depolarized activation threshold than other channels. SNS is found preferentially in C-fibers of peripheral nerves. Several lines of evidence suggest that this channel is important in the transmission of nociceptive information to the spinal cord. To test the hypothesis that SNS has a specialized role in pain sensation, and to examine its role in nociception, we generated a null-mutant mouse for SNS. We found that such mice are normal, apart from partial deficits in perception of noxious thermal, mechanical and inflammatory stimuli.

METHODS

Gene targeting

129Sv genomic DNA was used to construct a targeting vector. Genomic fragments containing a part of sns exon 4 (short arm) and exons 5–9 (long arm) were ligated into pBluescript that contained a PGK-neo cassette and a thymidine kinase dimer cassette (MC1) to give the targeting construct (Fig. 1).
E14-TG2a subclone IV cells were electroporated with NotI-linearized vector. Cells were selected with GM418 and gancyclovir, and three correctly targeted single-copy integrations were identified. Clones were injected into C57BL/6 blastocysts and subsequently implanted into CBA × C57BL/6 F1 foster mothers. Transmission of the targeted allele was confirmed by Southern blot analysis. Digests of tail DNA with EcoRI were Southern blotted and probed with a random-prime-labeled ApaI-EcoRI genomic fragment (Fig. 1). Null-mutant digests containing the PGK-neo cassette produce a band of 8.8-kb, compared to the 7.8-kb band found in wild-type animals.

**Electrophysiology**

We recorded from DRG neurons (1–5 days in culture) two hours after replating at room temperature. The cDNA encoding rat SNS in the expression vector pGW-1, together with the GFP expression vector pGW-1-GFP and 0.5% FITC-dextran were injected into neuronal nuclei. Recordings were made 1–2 days after injection using the whole-cell, patch-clamp technique. pClamp6 software (Axon) was used to acquire and analyze the currents. The pipet solution contained 130 mM CsCl, 10 mM NaCl, 1 mM MgCl₂, 10 mM HEPES and 5 mM EGTA, pH 7.35. The extracellular solution contained 105 mM cholineCl, 35 mM NaCl, 3 mM CsCl, 1 mM MgCl₂, 0.01 mM CaCl₂, 0.05 mM CdCl₂, 11 mM glucose and 5 mM HEPES, pH 7.4. In some experiments, 500 nM TTX was included in the extracellular solution. Calcium channel activity was blocked by the inclusion of CdCl₂ in the extracellular solution.

For electrophysiological studies in vivo, mice were anesthetized with urethane (3 g/kg, i.p.). Following a laminectomy, single neuron extracellular recordings were made using a tungsten electrode in the dorsal horn. Electrical stimulation (2 ms pulse) in the peripheral receptive field evoked a short-latency, low-threshold response (<10 ms after stimulus) and a longer-latency, high-threshold response (100–200 ms after stimulus) in the dorsal horn neuron, which reflect recruitment of A- and C-fibers, respectively.

**Behavioral studies**

Mice were examined for spinal reflexes and motor skills as described in detail elsewhere. Nociceptive thresholds to thermal stimuli were determined using
Fig. 2 Sodium currents recorded in DRG neurons from \textit{sns}-null mutant mouse. Currents were evoked by step depolarizations to \(-50\) to \(20\) mV and superimposed. \textbf{A}, control recordings evoked from a holding potential of \(-100\) mV in the absence of tetrodotoxin (TTX). \textbf{B}, in the presence of \(2 \times 10^{-7}\) M TTX. \textbf{C}, a holding potential of \(-80\) mV in the absence of TTX. \textbf{D}, a holding potential of \(-50\) mV in the absence of TTX.

hot-plate, paw-flick (method of Hargreaves) or tail-flick methods. For the hot plate, the latency for the mouse to lick its hindpaw or jump was recorded. Paw-flick latencies were determined for both hind paws on three occasions. Tail-flick latencies were determined on three occasions. Mechanical sensitivity was determined using calibrated Von Frey hairs. Hairs were applied three times each in ascending order of force, through a mesh floor, until a response was elicited (paw withdrawal, shaking or biting). Percent responses were calculated for each hair. Nociceptive thresholds to noxious mechanical stimuli were determined using a Ugo Basile algesiometer. The tail was gradually compressed until an escape response was elicited (biting the apparatus, vocalization or struggle). Carrageenan-induced-thermal hyperalgesia was assessed\(^9\) using an intraplantar injection of 20 \(\mu\)l of carrageenan (0.6 mg) or saline into one hind paw.

**RESULTS**

Voltage-gated sodium channel \(\alpha\) subunits contain S4-domain voltage sensors that are essential for activity\(^{13}\). We therefore generated a targeting construct by substituting for exons 4 and 5 of \textit{sns}, which include the S4 voltage sensor of domain I (Fig. 1), with a PGK-neo cassette terminating with stop codons in all three frames\(^6,12\). Null mutant mice were healthy, fertile and indistinguishable in their appearance, spontaneous behavior, body weight and body temperature from age- and sex-matched, wild-type control animals.

As shown in Fig. 2, only the fast sodium currents were evoked from a holding potential of \(-100\) mV (A) in \textit{sns}-null mutants. These currents were completely blocked by a low concentration of TTX, indicating that SNS is absent (B). This could be confirmed also electrophysiologically. When the holding potential...
was change to more depolarized level of \(-80\) mV (C), the current amplitude became reduced, and at \(-50\) mV holding potential, inward current was totally disappeared (D). These findings are consistent with the inactivation kinetics for TTX-sensitive fast sodium currents\(^9\). \textbf{Fig. 3} further demonstrates the absence of SNS in null mutant mice. Sodium current recorded from null mutants (A) had the time course identical with that of the TTX-sensitive current recorded from wild type mice but much faster than that of SNS (B-2). These experiments confirm that SNS is responsible for the slow TTX-resistant currents found in sensory neurons.

All null-mutant and wild-type mice exhibited normal placing and righting reflexes and hindlimb reflex extension, and they had no difficulty in maintaining their balance on beams for the full 120-second trial duration. Similarly, null-mutant mice were able to maintain their balance on a revolving rotorod for the same duration as wild-type mice\(^1\). Spontaneous locomotor activity was not different in sns null-mutant and wild-type mice.

The paw-withdrawal and tail-flick latencies following exposure to a noxious thermal (radiant heat) stimulus were significantly increased, albeit to a small extent, in null mutants compared with wild-type mice (\textbf{Fig. 4A and B}). In contrast, there was no difference in the latency on the hot plate of null-mutant and wild-type mice across a range of temperatures. Pain thresholds to noxious mechanical stimuli applied to the tail were markedly increased in the null-mutant mice (\textbf{Fig. 4C}), which all went to the cutoff point of the test, demonstrating a pronounced mechanical analgesia. In contrast, paw-withdrawal responses elicited by graded Von Frey hairs did not differ between null-mutant and wild-type mice (\textbf{Fig. 4D}).

TTX-resistant sodium channels may be involved in persistent inflammatory pain states\(^2,3,9\). In wild-type mice, intraplantar injection of carrageenan induced thermal hyperalgesia. Hyperalgesia was apparent at one hour and was maximal for several hours following injection. Thermal hyperalgesia developed more slowly in null-mutant mice, with significant hyperalgesia being observed at two and three hours. However, the maximum level of hyperalgesia was not significantly different in wild-type and null-mutant mice (\textbf{Fig. 4E}).

\textbf{DISCUSSION}

Small-diameter sensory neurons express TTX-resistant sodium channels\(^7,8\), but the absence of subtype-specific sodium channel blockers has precluded a study of the role of individual channel subtypes in nociception. Using gene-targeting technology to delete the TTX-resistant sodium channel
we find evidence to suggest that there is a selective role for this channel in pain sensation. The deletion of sns leads to the loss of all slow TTX-resistant currents in sensory neurons. However, electrophysiological studies have demonstrated two or more types of TTX-resistant current in DRG neurons. These apparently contradictory observations can be reconciled if distinct accessory subunits exist that modify the functional properties of α subunits in a manner analogous to the previously identified sodium channel β subunits.

Behavioral studies demonstrate a clear-cut deficit in both mecanano- and thermoreception and a temporarily diminished response to inflammatory pain stimuli in null-mutant animals. Inflammatory hyperalgesia evoked by prostaglandin E2 is reversibly blocked by antisense oligonucleotides directed against sns. The less-pronounced analgesic phenotype demonstrated in sns null mutant mice may reflect developmental compensatory mechanisms. The delayed development of inflammatory hyperalgesia suggests that targets other than SNS have a later cooperative role in the development of hyperalgesia. The partial analgesia shown by null mutants is consistent with observations about the role of TTX-resistant currents in nociception. These observations, combined with other indirect evidence that the regulation of TTX-resistant channels is important in inflammatory pain states, suggest that blockers of SNS synthesis or activity should be specific analgesics.

References


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