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SMALL INTERFERING RNA-MEDIATED SELECTIVE KNOCKDOWN OF $Na_V1.8$ TETRODOTOXIN-RESISTANT SODIUM CHANNEL REVERSES MECHANICAL ALLODYNIA IN NEUROPATHIC RATS

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Abstract—The biophysical properties of a tetrodotoxin resistant (TTXr) sodium channel, $Na_V1.8$, and its restricted expression to the peripheral sensory neurons suggest that blocking this channel might have therapeutic potential in various pain states and may offer improved tolerability compared with existing sodium channel blockers. However, the role of $Na_V1.8$ in nociception cannot be tested using a traditional pharmacological approach with small molecules because currently available sodium channel blockers do not distinguish between sodium channel subtypes. We sought to determine whether small interfering RNAs (siRNAs) might be capable of achieving the desired selectivity. Using Northern blot analysis and membrane potential measurement, several siRNAs were identified that were capable of a highly-selective attenuation of $Na_V1.8$ message as well as functional expression in clonal ND7/23 cells which were stably transfected with the rat $Na_V1.8$ gene. Functional knockdown of the channel was confirmed using whole-cell voltage-clamp electrophysiology. One of the siRNA probes showing a robust knockdown of $Na_V1.8$ current was evaluated for *in vivo* efficacy in reversing an established tactile allodynia in the rat chronic constriction nerve-injury (CCI) model. The siRNA, which was delivered to lumbar dorsal root ganglia (DRG) via an indwelling epidural cannula, caused a significant reduction of $Na_V1.8$ mRNA expression in lumbar 4 and 5 (L4–L5) DRG neurons and consequently reversed mechanical allodynia in CCI rats. We conclude that silencing of $Na_V1.8$ channel using a siRNA approach is capable of producing pain relief in the CCI model and further support a role for $Na_V1.8$ in pathological sensory dysfunction.

Key words: dorsal root ganglia, CCI, pain, gene expression, sodium current, ND7/23 cell.

Voltage-gated sodium channels have long been recognized as being critically important for the initiation and propagation of action potentials in neurons. Nine sodium channel genes have been identified with sufficient sequence homology to justify their consideration as a single family cluster ($Na_V1.1$–1.9; see Catterall et al., 2003). Six of the nine channel variants are highly sensitive to the prototypic sodium channel toxin, tetrodotoxin (TTX), the remaining three family members, $Na_V1.5$, $Na_V1.8$ and $Na_V1.9$, are unusual in their variable but relative insensitivity to TTX. Sodium channels, in general, represent interesting therapeutic targets and, indeed, a number of successful drugs have been developed that owe their therapeutic efficacy to potent sodium channel block (e.g. local anesthetics, anticonvulsants, antiarrhythmics). The utility of several of these drugs in the clinical management of chronic pain has been repeatedly demonstrated (for review see Priestley and Hunter, 2006). However, none of the currently used therapeutics is capable of distinguishing between sodium channel isoforms and several adverse events, attributed to the resulting broad-spectrum sodium channel block, have severely undermined patient compliance when these medications are used in a pain setting.

The challenge facing novel sodium channel drug discovery in the pain arena is to identify the primary transcripts responsible for nociceptive signaling and to selectively block such abnormal pathological activity in sensory afferent nerves. The $Na_V1.8$ transcript is one of several channel isoforms that is particularly interesting with respect to sensory nerve pathophysiology because it is only expressed in a subset of primary afferent nerves (Akopian et al., 1996; Sangameswaran et al., 1996) and it has been linked to various pain states (see reviews by Lai et al., 2004; Priestley, 2004). However, restricted expression alone does not obviate the need for selective pharmacology and this is graphically illustrated in the case of the sensory nervous system where individual afferents express several sodium channel transcripts in addition to $Na_V1.8$. To address the issue of specificity, various groups have adopted reverse-genetics strategies, typically using either antisense oligodeoxynucleosides (ODNs) to disrupt post-transcriptional efficiency or, alternatively, gene-knockout approaches. For example, $Na_V1.8$-specific ODNs have been shown to reduce the hyperalgesia provoked by intraplantar injections of either prostaglandin E2 (PGE2; Khasar et al., 1998; Villarreal et al., 2005) or complete Freund's adjuvant (CFA; Porreca et al., 1999). The same approach has also been applied to neuropathic

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Abbreviations: CCI, chronic constriction nerve injury; CFA, complete Freund's adjuvant; DRG, dorsal root ganglia; I/V, current–voltage; L6 (4, 5), 6th (4th, 5th) lumbar; mm-siRNA, mismatch small interfering RNA; ns-siRNA, non-silencing small interfering RNA; ODN, oligodeoxynucleoside; PGE2, prostaglandin E2; PWT, paw withdrawal threshold; siRNA, small interfering RNA; TTX, tetrodotoxin; TTXr, tetrodotoxin-resistant; TTXs, tetrodotoxin-sensitive; V_h, membrane holding potential; wt, wild type.

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pain models and convincing ODN-mediated efficacy has been demonstrated in both the spinal nerve ligation (Porreca et al., 1999) and chronic constriction injury (CCI) models (Liu et al., 2005). In each case, efficacy correlated with a corresponding reduction in $Na_V1.8$ mRNA levels. Gene deletion studies have produced a less convincing picture as regards the role of $Na_V1.8$ in chronic pain. An initial phenotypic characterization of a $Na_V1.8$ knockout mouse revealed a delayed and marginally-blunted hyperalgesic response to intraplantar CFA (Akopian et al., 1999). Subsequent studies, using the same mice, suggested a requisite role for the channel in nerve growth factor–induced hyperalgesia but not in PGE2-induced inflammatory thermal hyperalgesia and only an equivocal role in neuropathic pain resulting from a partial sciatic nerve injury (Kerr et al., 2001).

Each of the above genetic approaches has its strengths and weaknesses. Gene knockout is frequently criticized because of potential developmental issues and the $Na_V1.8$-null mouse was, indeed, associated with a compensatory increase in the $Na_V1.7$ sodium channel transcript (Akopian et al., 1999). Antisense ODNs have been used extensively as research tools but they suffer from variable silencing efficacy and off-target effects (Dorsett and Tuschl, 2004; Rohl and Kurreck, 2006) which complicate the therapeutic application of this technology. In the present study, we sought to examine the potential of a small interfering RNA (siRNA) approach that exploits a nucleic-acid-based target-selectivity advantage to achieve specific gene silencing (Milhavet et al., 2003). The siRNA approach has been demonstrated to be more tractable than antisense ODN, both as an investigative tool and as a potential therapeutic approach (see Dorsett and Tuschl, 2004 for a comparative review). For example, the siRNA approach has been reported to confer high levels of sequence-specific mRNA degradation of several therapeutically relevant genes, both *in vitro* and *in vivo*, without major off-target liabilities. Furthermore, siRNA sequences can be designed into short hairpin structures that can be packaged in viral vectors and delivered to target tissues by infection strategies (Azkur et al., 2005; Hong et al., 2006) that have the potential to yield long-lasting gene silencing.

In this study, several putative siRNAs designed against the rat $Na_V1.8$ coding sequence (siRNA-$Na_V1.8$) were evaluated for their ability to selectively attenuate the $Na_V1.8$ message as well as the functional expression in a clonal ND7/23 cell line which was stably transfected with the rat $Na_V1.8$ gene. To determine their *in vivo* efficacy in regulating $Na_V1.8$ expression in dorsal root ganglia (DRGs) and relieving neuropathic pain, one of the effective siRNAs was delivered via an indwelling epidural cannula to lumbar DRGs, in rats that had previously been subjected to a CCI to the left sciatic nerve. The effect on tactile allodynia in CCI rats was evaluated and subsequently the $Na_V1.8$ expression level in the 4th and 5th lumbar (L4 and 5) DRGs was determined.

EXPERIMENTAL PROCEDURES

Animals

Male adult Wistar rats (150–250 g) were used in the study. Animals were housed in groups of three (but individually for 1 week after surgery) in plastic cages with free access to food and water under a 12-h light/dark cycle. Animals were acclimated for 1 week before experiments. All experimental procedures were approved by the Schering-Plough Research Institute Animal Care and Use Committee and were in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain. All efforts were made to minimize the number of animals used and their suffering.

Surgery

For all surgical procedures, the animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally for induction, and 15–20 mg/kg every hour for maintenance). An adequate depth of anesthesia was monitored, at regular intervals, by confirming absence of responses to noxious stimulation. Rectal temperature was maintained near 37 °C by a controlled heating blanket. Surgeries for epidural catheterization and nerve injury were performed using the procedures described below. Upon completion of the surgery, hemostasis was confirmed and the incisions were closed in layers with suture. The rats were kept warm during recovery from anesthesia, after which they returned to their home cage in the animal colony. Surgically operated rats were inspected daily for signs of autotomy and apparent paralysis. Their body weight, food and water intake were also monitored. On rare occasions, early signs of autotomy were seen (gnawing of claw tips and some surrounding tissue on the injured hind paw), and the animal was promptly killed.

CCI of the sciatic nerve model. A CCI of the sciatic nerve was induced according to the method of Bennett and Xie (1988). Briefly, following a skin incision made on the lateral surface of the left thigh, the common sciatic nerve was exposed at mid-thigh level by blunt dissection through the biceps femoris muscle. Proximal to the trifurcation, a 10 mm portion of nerve was freed of adhering tissue and four loose ligatures (4.0 chromic gut suture) were tied around the sciatic nerve (1–1.5 mm apart).

Epidural catheterization. The catheterization of the lumbar epidural space was carried out in the CCI rats on post-operative days 7–8 using a modified procedure of a previously reported method by van den Hoogen and Colpaert (1981). Briefly, rats were placed in the prone position and a small midline incision was made in the region of the 1st to 3rd lumbar vertebrae. Following the superficial muscles being carefully dissected and laterally retracted, a small hole was made in the intervertebral space between L1–L2. A polyethylene catheter (PE-10; nominal i.d. 0.28 mm, length 26 cm) was then inserted to the lumbar epidural space and gently advanced about 2–2.5 cm caudally so that the catheter tip reached the region of L4 vertebra. The catheter was secured with a 4–0 silk suture tied to a 0.5 mm hole made in the L1 spinous process. Animals were excluded if blood or cerebrospinal fluid was aspirated. The remainder of the catheter was subcutaneously tunneled and exited through a small incision at the back of the neck. The catheter was blocked with a metal plug and secured to the skin with a suture. At the end of the procedure the catheter was flushed with 15 μl sterile saline, a volume equivalent to the volume of the catheter. Buprenorphine (0.05–0.1 mg/kg, s.c.) was applied to the surgical site on the completion of the surgery to provide post-surgical pain relief. To confirm correct catheter positioning, 50 μl of 2% lidocaine was administered through the catheter after complete recovery from anesthesia. A correct epidural catheter placement was judged by paralysis of the hindlimbs without affecting normal forelimb motor function. The