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Silencing of the $Ca_V3.2$ T-type calcium channel gene in sensory neurons demonstrates its major role in nociception

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Analgesic therapies are still limited and sometimes poorly effective, therefore finding new targets for the development of innovative drugs is urgently needed. In order to validate the potential utility of blocking T-type calcium channels to reduce nociception, we explored the effects of intrathecally administered oligodeoxynucleotide antisenses, specific to the recently identified T-type calcium channel family ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$), on reactions to noxious stimuli in healthy and mononeuropathic rats. Our results demonstrate that the antisense targeting $Ca_V3.2$ induced a knockdown of the $Ca_V3.2$ mRNA and protein expression as well as a large reduction of '$Ca_V3.2$-like' T-type currents in nociceptive dorsal root ganglion neurons. Concomitantly, the antisense treatment resulted in major antinociceptive, anti-hyperalgesic, and anti-allodynic effects, suggesting that $Ca_V3.2$ plays a major pronociceptive role in acute and chronic pain states. Taken together, the results provide direct evidence linking $Ca_V3.2$ T-type channels to pain perception and suggest that $Ca_V3.2$ may offer a specific molecular target for the treatment of pain.

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Introduction

Management of pain is an essential aspect of modern medicine and more globally for the quality of life; however, current therapies are frequently insufficient owing to severe side effects or limited effectiveness. Therefore, the discovery of new analgesics is needed, especially to treat the proportion of painful patients poorly improved by available analgesics (e.g. neuropathic pain patients). Basic research into the molecular mechanisms of pain transmission and perception will help to better describe the fundamental knowledge of nociceptive pathways. Recent work has shown that specific sets of ion channels and transmembrane receptors appear largely confined to primary afferent neurons where they likely contribute to the detection and transmission of different kinds of nociceptive stimuli (Julius and Basbaum, 2001). Moreover, remodelling of their expression pattern is reported in different animal models of neuropathic or inflammatory pain (Scholz and Woolf, 2002). Many of these nociceptor-specific membrane proteins represent promising targets for the development of new analgesics expected to possess a better benefit/risk ratio compared to current therapies.

While a number of ionic conductances contribute to neuronal firing, voltage-gated calcium channels are unique in being involved in both shaping the action potential and triggering downstream physiological responses such as neurotransmitter release and calcium-dependent gene expression. A total of 10 pore-forming calcium channel α_1 subunits have been identified (Ertel *et al*, 2000), including the low-voltage activated (LVA) T-type calcium channel family ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) activated by weak depolarizations above resting potential and considered as efficient tuners of cell excitability (Huguenard, 1996; Chemin *et al*, 2002). Understanding the physiological roles of T-type calcium channels has been limited by the lack of selective pharmacology (Perez-Reyes, 2003). Nonetheless, T-type channels have been functionally described in dorsal root ganglion (DRG) neurons, suggesting a possible role in sensory perception and nociception (Carbone and Lux, 1984; Bossu *et al*, 1985; Nowycky *et al*, 1985; Scroggs and Fox, 1992). More recently, reverse transcription (RT)–PCR and *in situ* hybridization analyses have shown that a subset of small- and medium-diameter primary afferent neurons as well as neurons from the superficial laminae of the dorsal horn express $Ca_V3.2$ almost exclusively (Talley *et al*, 1999; Shin *et al*, 2003). Furthermore, a mechanoreceptor subpopulation (D-hair cells) distinct from polymodal nociceptors but possibly implicated in allodynic behaviour expresses the highest density of $Ca_V3.2$ (Shin *et al*, 2003). The large T-type currents in these neurons are essential for light touch perception (Dubreuil *et al*, 2004). T-type channels are also essential for long-term potentiation of synaptic transmission between nociceptive primary afferents and superficial laminae substance-P-sensitive neurons of the dorsal horn (Ikeda *et al*, 2003). Consistent with this notion, a pronociceptive role of these channels at peripheral and spinal levels was suggested using weakly selective T-type channel antagonists in healthy and neuropathic animals (Matthews

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and Dickenson, 2001; Todorovic *et al*, 2002; Doğrul *et al*, 2003; Kim *et al*, 2003).

With the identification of the Ca_V3 gene family, genetic elimination of T-type isoforms has been possible, and mice lacking the $Ca_V3.1$ and $Ca_V3.2$ isoforms have been reported (Kim *et al*, 2001b; Chen *et al*, 2003). Peripheral pain behaviour was shown to be unaffected in the $Ca_V3.1$ knockout (KO) mice and remains to be explored in the $Ca_V3.2$ KO mice. However, a general gene knockout approach is often limited by developmental problems and by compensatory mechanisms. This was notably shown for the nociceptor-specific TTX-resistant sodium channel $Na_V1.8$ KO (Akopian *et al*, 1999). Waiting for more sophisticated genetic strategies featuring inducible and localized knockouts in nociceptors, intrathecal oligonucleotide (ODN) antisense (AS)-mediated knockdown has been shown to be a reliable alternative to produce clear phenotypes (Lai *et al*, 2002; Stone and Vulchanova, 2003). In the present study, we have used such a targeted AS-ODN knockdown strategy to explore the functional contribution of T-type calcium channel isoforms to the physiology and pathophysiology of peripheral pain transmission. Our results demonstrate that $Ca_V3.2$ has a major pronociceptive role and represents a promising new target for the development of specific analgesic drugs.

Results

Molecular analysis of the $Ca_V3.2$ knockdown

We used AS oligodeoxynucleotides (ODNs) to knock down the genes encoding the T-type calcium channel pore-forming α_1 subunits. We administered either a generic AS sequence (AS-Ca_V3-com) targeting all three members of this gene family, or three specific AS-ODNs complementary to each $Ca_V3.x$ subtype (AS-$Ca_V3.1$, AS-$Ca_V3.2$, and AS-$Ca_V3.3$) (see Materials and methods).

To estimate the level of ODN uptake by the different neuronal structures following the intrathecal (i.t.) injection, the AS AS-Ca_V3-com, the AS-CaV3.2, and the Mismatch-2 ODN were labelled with a fluorescent group (FITC). After dissection of the tissues without fixation of the animals ($n=3$, AS-$Ca_V3.2$-FITC), we observed that the fluorescent-ODN readily penetrated the lumbar DRGs near the i.t. injection site (Figure 1A). However, at upper levels, thoracic DRGs were not affected as shown by the lack of FITC labelling (Figure 1B). Regarding the spinal cord, examination of the lumbar segment showed that most of the fluorescence remained trapped in the surrounding meninges (Figure 1C) with much less penetration into the spinal cord compared to the delivery into the lumbar DRGs (compare Figure 1A and C). Similar tissue deliveries were obtained with the two other FITC-ODNs (not shown).

To demonstrate the successful AS-mediated knockdown of T-type channels, we analysed the expression of $Ca_V3.2$ mRNA and protein in DRG and spinal cord from saline-, mismatch-, and AS-$Ca_V3.2$-injected rats, taking into account that ODNs are prominently delivered to DRGs known to express almost exclusively the $Ca_V3.2$ isotype. The $Ca_V3.2$ mRNA levels in the various tissues, analysed by quantitative reverse transcription (QRT)–PCR amplification, are shown in Figure 1D ($n=8$–11). QRT–PCR results showed a $Ca_V3.2$ mRNA decrease (-42%, $P<0.001$) exclusively in lumbar DRGs of AS-$Ca_V3.2$-treated animals. No significant change was observed in either the thoracic DRGs distant from the AS injection site, or in the lumbar spinal cord in the vicinity of the injection site. Furthermore, determination of the mRNA abundance of the two other T-type calcium channel genes in the lumbar DRGs showed a very low expression of $Ca_V3.1$ and $Ca_V3.3$ that was not influenced by the AS-$Ca_V3.2$ treatment (Figure 1D, $n=3$). All the values are expressed as relative $Ca_V3.x$ amounts compared to HPRT levels, and similar results were obtained using synaptophysin as a second housekeeping gene (not shown).

The $Ca_V3.2$ knockdown was further analysed at the protein level. We first characterized anti-$Ca_V3.2$ antibodies (Ab) with the help of the $Ca_V3.2$ KO mice by immunoblot analysis of DRG proteins of wild-type (WT) and KO animals. As depicted in Figure 1E, a Western blot analysis with an Ab raised against a fusion protein of the rat-$Ca_V3.2$ sequence revealed a specific band around 230 kDa in DRG samples obtained from WT mice but not from $Ca_V3.2$ KO mice. This Ab also recognizes an aspecific lower band around 170 kDa in samples from both WT and $Ca_V3.2$ KO mice. Analysis of spinal cord proteins revealed the same banding pattern. In addition, analysis of HEK cell membranes expressing each recombinant Ca_V3 channel confirmed the specific immunoreactivity of the Ab for $Ca_V3.2$ with no crossreactivity with $Ca_V3.1$ or $Ca_V3.3$. This staining was abolished by preabsorption of the Ab with the immunizing fusion protein (J McRory and TP Snutch, unpublished data).

When immunoblotted on proteins from AS-$Ca_V3.2$-, mismatch ODN- or saline-injected rats at the end of the treatment, the anti-$Ca_V3.2$ Ab revealed that the 230 kDa immunoreactivity was strongly reduced in lumbar DRG extracts but not in the thoracic DRG level (Figure 1F), nor in the spinal cord (not shown). In all cases, the aspecific 170 kDa band as well as the immunodetection of β-tubulin (Figure 1E and F) or Erk2 (not shown) served as loading controls. Similar results were obtained on four immunoblots using protein samples from two distinct experiments.

Antisense to $Ca_V3.2$ reduces T-type currents in nociceptors

In order to provide a functional evidence of the $Ca_V3.2$ knockdown, the T-type calcium current density was compared in dissociated nociceptive DRG neurons from rats injected either with AS-$Ca_V3.2$, the mismatch ODN, or the vehicle. We used the cell body size to distinguish nociceptive neurons within the DRG cell populations obtained after dissociation using an eyepiece scale on the microscope objective. We recorded small (15–30 μm diameter) and medium (30–40 μm) neurons that correlate well with unmyelinated (C) and thinly myelinated (Aδ) fibre groups, including the majority of nociceptors. Small and medium neurons recorded from each animal group had similar mean capacitance (in pF: saline: small (S) 38 ± 2, $n=17$, medium (M) 70 ± 4, $n=26$; mismatch: S 39 ± 1, $n=26$, M 67 ± 4, $n=15$; AS-$Ca_V3.2$: S 36 ± 1, $n=38$, M 76 ± 7, $n=15$). Calcium channel activity was measured using a nearly physiological level of external calcium (2 mM). Currents were evoked by depolarizations of increasing amplitude from a negative holding potential (HP, -100 mV). Different electrophysiological profiles were found according to the expression of T-type currents, which were present in 42 and 66% of small- and medium-sized control neurons tested, respectively. The

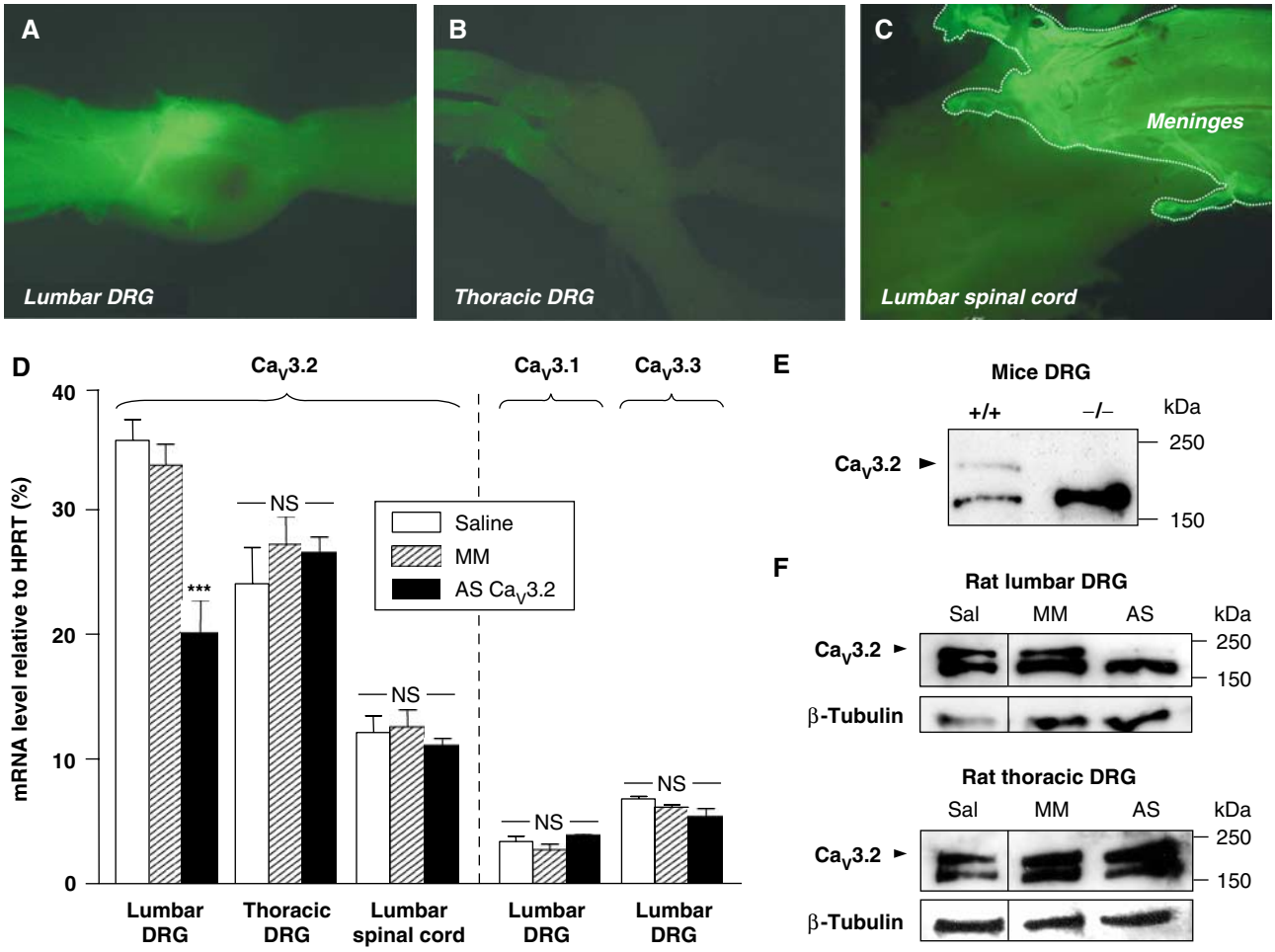

Figure 1 I.t. administration of $Ca_V3.2$-AS leads to a preferential uptake of AS in lumbar DRGs leading to a selective knockdown of $Ca_V3.2$ protein levels. After the last injection of AS-$Ca_V3.2$-FITC, bright fluorescence can be detected in freshly dissected lumbar DRG (**A**). In comparison, the thoracic DRGs did not show FITC staining (**B**). (**C**) In the lumbar enlargement near the ODN injection site, most of the FITC signal was retained in the meninges. The picture shows a dorsal view of the spinal cord with the meninges partially unfolded on the left side and still attached to the spinal cord on the right side. Note that a dotted line has been added to the border of the floating meninges to delineate the edge (C). Note that compared to the lumbar DRG, weaker levels of FITC fluorescence were seen on the spinal cord. (**D**) QRT–PCR analysis of $Ca_V3.2$ transcripts in lumbar and thoracic DRGs and in lumbar spinal cord of saline-, mismatch-, and AS-$Ca_V3.2$-treated rats ($n=8$ –11). Comparative analysis of $Ca_V3.1$ and $Ca_V3.3$ in lumbar DRGs of the same animal groups ($n=3$). (**E**) A rabbit Ab raised against a rat $Ca_V3.2$ fusion protein recognizes a 230 kDa band in DRG membranes (40 μg/lane) from WT but not from $Ca_V3.2$ KO mice (representative blot of three independent experiments). (**F**) Western blots of rat DRG membrane proteins with the anti-$Ca_V3.2$ Ab. Analysis of $Ca_V3.2$ in DRG extracts after saline, mismatch, or AS AS-$Ca_V3.2$ treatment. In each case, equal amounts of membrane proteins were loaded on 4–20% gradient polyacrylamide gels, subjected to SDS–PAGE, and transferred to nitrocellulose as described in Materials and methods. The apparent molecular size markers are indicated on the right. Blots are representative of four independent experiments. Note that the AS treatment induces a strong reduction of the $Ca_V3.2$ immunoreactivity in the lumbar DRGs (see arrowhead in (F)). Loading controls were provided by immunoblotting β-tubulin protein.

activation threshold of T-type calcium currents was near −70 mV and the maximum current was evoked at −40 mV. In contrast, high-voltage activated (HVA) currents started to be evoked by potential above −35 mV, and peaked between 0 and +10 mV. The T-type currents evoked at −40 mV were almost completely inactivated at an HP of −60 mV showing that, at this test depolarisation, the HVA channel activity does not contribute to the recorded calcium (Supplementary data). As previously documented, T-type currents in both small- and medium-sized DRG neurons exhibited '$Ca_V3.2$-like' functional properties. In particular, both DRG and recombinant $Ca_V3.2$ T-type currents were potently blocked by nickel (10 μM Ni^{2+}: 78% block, $n=7$). As shown in Figure 2A, the AS-$Ca_V3.2$ treatment resulted in a dramatic reduction of T-type currents (illustrated for two small-sized neurons). The current density in the AS group dropped by 75 and 92% respectively in small and medium neurons (Figure 2B; in pA/pF: saline: S 2±0.4, M 14.6±4.1; mismatch: S 2.4±0.8, M 13.4±5.2; AS-$Ca_V3.2$: S 0.6±0.1, M 1±0.2). The remaining current in the AS-treated neurons had similar properties to the currents recorded in the mismatch and the saline groups. Comparison of the T-type currents activation and inactivation kinetics at −40 mV showed no differences between conditions (τ_{act} (s): control: S 4.9±1.1, $n=10$, M 5.2±0.8, $n=9$; mismatch: S 4.8±0.8, $n=6$, M 5±1.2, $n=5$; AS-$Ca_V3.2$: S 5.1±0.8, $n=7$, M 5.4±1.1, $n=4$; τ_{inact} (s): control: S 26.3±4.8, $n=10$, M 27.4±6, $n=9$; mismatch: S 24.7±5.5, $n=6$, M 26.1±5.8, $n=4$; AS-$Ca_V3.2$: S 28.2±5.2, $n=7$, M 27.1±4, $n=4$). Finally, HVA currents were systematically recorded in each cell and no effect of AS-$Ca_V3.2$ was observed

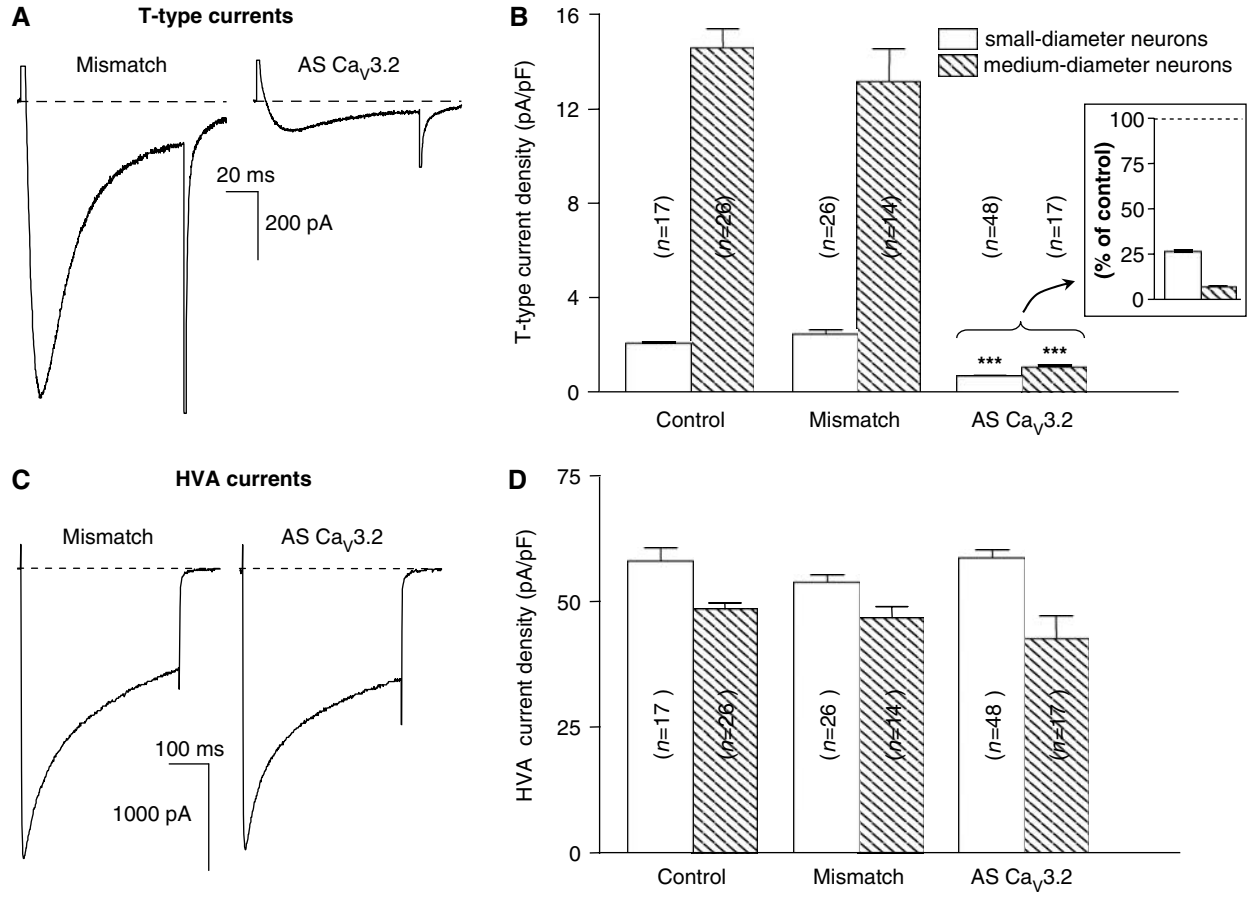

Figure 2 The AS treatment against the $Ca_V3.2$ subunit induces a reduction of T-type calcium currents in isolated lumbar DRG neurons. (**A**) Representative T-type calcium currents evoked by 100 ms long depolarizations from −100 to −40 mV in small nociceptive DRG neurons isolated from mismatch- or AS-$Ca_V3.2$-injected rats. Note the reduced size of the current in the AS-treated cell. (**B**) Averaged peak T-type current density (mean±s.e.m.) recorded from small- and medium-sized isolated neuron cell bodies from mismatch-, AS-, or saline-treated animals. Note that the AS treatment results in a highly significant (***$P<0.001$) knockdown of the T-type channel activity in both small- and medium-diameter neurons as illustrated in the inset. (**C**) Representative traces of ensemble HVA calcium currents evoked by 400 ms long depolarizations from −100 to +10 mV in nociceptive DRG neurons isolated from mismatch- or AS-$Ca_V3.2$-injected rats. (**D**) Averaged peak HVA current density (mean±s.e.m.) recorded from the same cells showed on the histogram of part B. Note that the current density levels were not altered by the treatment. Cells were prepared from four animals in each group.

on the current kinetics (Figure 2C) and density (Figure 2D; in pA/pF: saline: S 58±9, M 48±6; mismatch: S 54±8, M 47±7; AS-$Ca_V3.2$: S 59±8, M 42±12).

Silencing of $Ca_V3.2$ strongly reduces acute and neuropathic nociception

The functional consequences of the local $Ca_V3.2$ knockdown in lumbar DRGs to noxious stimuli perception were evaluated *in vivo*. We first studied the effects of the generic AS (Figure 3) and then the specific AS (Figure 4). No obvious difference was observed in general behaviour of the animals between the different groups (AS-$Ca_V3.x$, mismatches, and salines), indicating the absence of deleterious nonspecific side effects of ODNs. The animals were visually undistinguishable between batches, they had similar food intake, and scoring of their locomotor activity showed no statistical differences (see Supplementary data). In contrast, a large and significant increase in vocalization threshold or delay of tail withdrawal to evoked noxious acute mechanical or thermal stimuli was observed in AS-Ca_V3-com-treated healthy animals, indicative of a strong antinociceptive effect (maximal increase: +351 ±75 g and +10.1±3.4 s, respectively) (Figure 3A and B). In addition, this effect was sustained and prolonged 4 to 5 days after the end of the treatment, respectively. No difference was noted between animals injected with a mismatch ODN and those injected with a saline solution. Since perception and integration of pain is largely amplified in neuropathic conditions, we tested the effects of AS-Ca_V3-com in rats subjected to the chronic constriction injury (CCI) model (Bennett and Xie, 1988). As illustrated in Figure 3C and D, the mechanical threshold and thermal latency were lowered (−149±5 g and −2.7±0.6 s, respectively), indicative of a hyperalgesic state 7 days after the ligature. AS-Ca_V3-com reversed completely the CCI-induced hyperalgesia and produced a large increase of the nociceptive threshold or latency (maximal increase: +446±67 g and +18.8±3.4 s, respectively) with a long duration of effect: 9- and 5-days, respectively (Figure 3A and C). This antinociceptive and antihyperalgesic efficacy was confirmed when the results were expressed as area under the time course curves (AUC) of variations in vocalization thresholds: 82 152±13 649 and 47 124±11 233 g·h, in CCI model and healthy animals, respectively ($P<0.05$). No significant difference was observed between mismatch AS- and saline-injected groups.

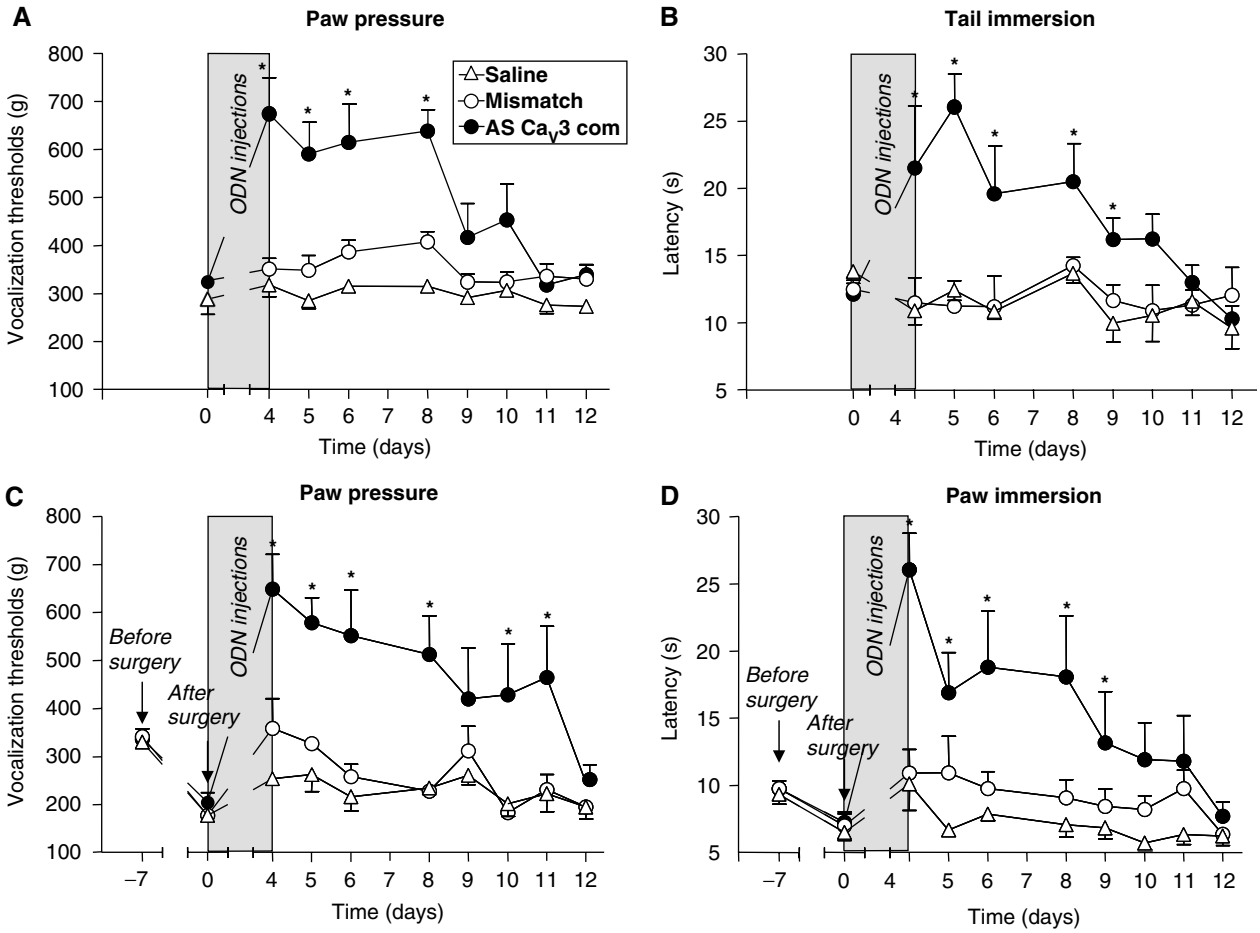

Figure 3 Time course of the effect of i.t. injection of a generic AS to Ca_V3 subunits on mechanical and thermal nociception in healthy and mononeuropathic rats (CCI model). (**A, B**) Three groups ($n = 5$–7) of animals were injected (i.t.) twice daily during 4 days with 10 µl containing either 12.5 µg of AS-Ca_V3-com (filled circles), 12.5 µg of the mismatch ODN (open circles), or the saline vehicle alone (open triangles). The rats were tested for nociceptive responses to a noxious pressure (A, paw pressure test) and to noxious thermal stimulus (B, tail immersion test). The response scale was measured in grams applied to the paw for the pressure test (vocalization thresholds) and in seconds for the latency of tail withdrawal from the water bath. Note that in both tests, the AS injection produced a 4-day significant antinociceptive effect. (**C, D**) For mononeuropathic rats, similarly, three groups of 5–7 animals were treated with AS-Ca_V3-com (filled circles), the mismatch ODN (open circles), and the saline vehicle (open triangles). Evoked mechanical (C) and thermal (D) hyperalgesia was scored using the paw pressure and the paw immersion tests. Thresholds were measured before (day −7) and after (day 0) the induction of neuropathy, then daily (days 4–12) after the end of the AS injection protocol. Note that the anti-hyperalgesic effect of AS-Ca_V3-com was prolonged in mononeuropathic animals compared to healthy rats. Each point represents the mean ± s.e.m. from 5–7 animals per treatment group. Statistical differences between measures $P < 0.05$ (*: AS-Ca_V3-com versus saline; ●: AS-Ca_V3-com versus mismatch) were calculated by ANOVA followed by a protected least significant difference (PLSD) Fischer *t*-test.

Further experiments were performed using specific AS-ODNs against each Ca_V3 gene family isoform. Since the generic AS induced qualitatively similar effects on both mechanical and thermal nociception, we only investigated the isoform-specific AS on the paw pressure test. The advantage of this test, based on the assessment of vocalization threshold, is to study a more integrated response. Interestingly, the results on healthy rats show that only the AS against the $Ca_V3.2$ subunit was able to recapitulate the antinociceptive effects of the generic AS, with a similar sustained analgesia lasting 4 days after the end of the treatment (maximal increase: $+248 \pm 50$ g) (Figure 4A). In contrast, neither the AS-$Ca_V3.1$ and the AS-$Ca_V3.3$, the mismatch, nor the saline injections evoked any significant modification of the vocalization threshold. AS-$Ca_V3.2$ when tested on mononeuropathic animals (Figure 4B) also reversed hyperalgesia and increased vocalization thresholds over preinjury scores (maximal increase: $+497 \pm 17$ g), reaching a plateau from day 4 to 9. AUC again showed a difference ($P < 0.001$) between healthy ($35\,145 \pm 6537$ g·h) and CCI ($72\,169 \pm 1565$ g·h) rats.

The influence of AS on neuropathic pain states was further assessed. In addition to altered responses to acute nociception, the CCI injury produced a stable state of tactile allodynia 14 days after surgery (mean threshold: 28.66 ± 0.11 g ($n = 22$) and 11.07 ± 0.13 g ($n = 22$) before and after surgery) (Figure 5) as previously described (Whiteside *et al*, 2004). Treatment with the saline or the mismatch ODN had no significant effect on tactile allodynia throughout the study. By contrast, the treatment with AS-$Ca_V3.2$ resulted in a large elevation (maximal increase: $+15.14 \pm 0.72$ g on day 5 after treatment) in paw withdrawal thresholds, reversing tactile sensitivity to the preinjury level. The effect of AS was reversible, the paw withdrawal thresholds returning to the allodynic range within 2 days after cessation of the i.t. injections.

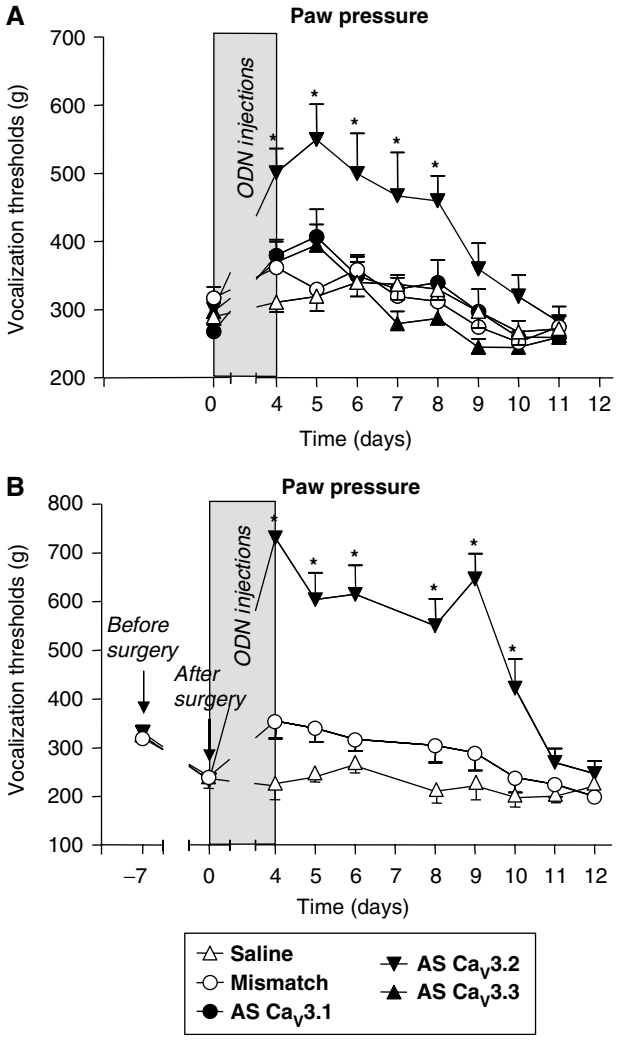

Figure 4 Selective effects of a specific AS to $Ca_V3.2$ on mechanical nociception. (**A**) Five groups of animals ($n=6$) were injected (i.t.) twice daily during 4 days with 10 µl containing either 12.5 µg of AS-$Ca_V3.1$ (filled circles), 12.5 µg of AS-$Ca_V3.2$ (filled down-triangles), 12.5 µg of AS-$Ca_V3.3$ (filled up-triangles), 12.5 µg of the mismatch ODN (open circles), or the saline vehicle alone (open up-triangles). The rats were tested for nociceptive responses to pressure (paw pressure test). The response scale was measured in grams applied to the paw (vocalization thresholds). Note that only the AS-$Ca_V3.2$ AS injection produced a significant antinociceptive effect. (**B**) For mononeuropathic animals, three groups of seven animals were treated either with AS-$Ca_V3.2$ (filled down-triangles), the mismatch ODN (open circles), or the saline vehicle alone (open up-triangles). Evoked mechanical hyperalgesia was scored as in panel A. Vocalization thresholds were measured before (day −7) and after (day 0) the induction of neuropathy, and then daily (days 4–12) after the end of the AS injection protocol. Each point represents the mean ± s.e.m. from 6–7 animals per treatment group. Statistical differences between measures $P<0.05$ (*: AS-$Ca_V3.2$ versus saline; ○: AS-$Ca_V3.2$ versus mismatch) were calculated by ANOVA followed by a PLSD Fischer *t*-test.

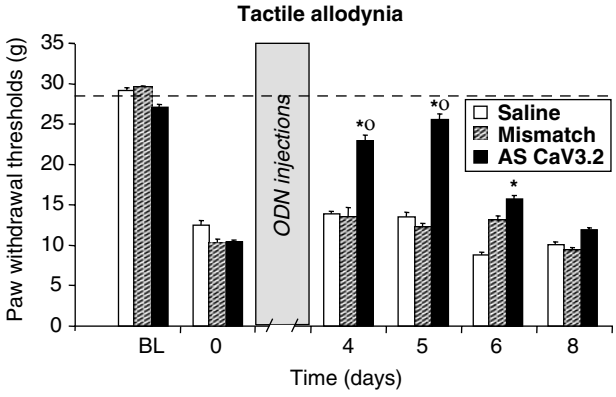

Figure 5 Effects of AS-$Ca_V3.2$ on tactile allodynia in mononeuropathic rats. Three groups of seven animals were injected (i.t.) twice daily during 4 days with 10 µl containing either 12.5 µg of AS-$Ca_V3.2$ (filled bars), 12.5 µg of the mismatch ODN (hatched bars), or the saline vehicle (open bars). Paw withdrawal thresholds were scored using the electronic von Frey Hair test. Thresholds were measured before injury (baseline values: BL) 14 days after the induction of neuropathy, before starting the injections (0), and then daily (days 4–8) after the end of the AS injection protocol. Each point represents the mean ± s.e.m. from seven animals per treatment group. Statistical differences between measures $P<0.05$ (*: AS-$Ca_V3.2$ versus saline; **o**: AS-$Ca_V3.2$ versus mismatch) were calculated by ANOVA followed by a PLSD Fischer *t*-test.

Discussion

This study represents the first direct demonstration of the role of T-type calcium channels in supporting peripheral nociception. While these channels have been proposed to play a role in sensory perception based on the effects of mibefradil *in vivo* (Todorovic *et al*, 2002; Doğrul *et al*, 2003; Kim *et al*, 2003), the largely questionable specificity of this molecule (Viana *et al*, 1997; Eller *et al*, 2000; Jimenez *et al*, 2000) precludes any conclusion on the implication of T-type calcium channel in nociception. Indeed mibefradil was shown to block potently other types of ion channels, including the N-type and R-type calcium channels, which have been clearly implicated in peripheral and spinal modulation of nociception (Kim *et al*, 2001a; Saegusa *et al*, 2000, 2001). In contrast, here we specifically inhibited the T-type calcium channel expression using an *in vivo* AS strategy with a repeated i.t. delivery, a method that has proven to be an efficient alternative to mouse knockout models, avoiding possible developmental compensatory problems (Stone and Vulchanova, 2003).

We have demonstrated that the direct transcutaneous lumbar i.t. injection of ODNs leads to an effective local and tissue-specific uptake of AS into the lumbar DRGs. Although AS-ODNs injected intrathecally were proven to act spinally (Hains *et al*, 2003), the spinal uptake of ODN is much less efficient, with most of the fluorescence restrained in the meninges, notably in the arachnoid/pia mater as previously reported (Rydh-Rinder *et al*, 2001).

The behavioural data presented here show that the generic AS targeting the $Ca_V3.x$ genes produced robust long-lasting and reversible mechanical and thermal antinociceptive effects in healthy animals, and elicited a marked anti-hyperalgesic effect on a mononeuropathic pain model. Interestingly, these effects were similar or greater in amplitude but of much longer duration than those of reference therapeutic analgesics, that is, morphine in healthy rats (Pelissier *et al*, 1996) and clomipramine in mononeuropathic rats (Marchand *et al*, 2003). More remarkably, the discrimination of the contribution of each of the T-type channel isoforms revealed that the antinociceptive effects are attributable to the unique repression of $Ca_V3.2$. Interestingly our data also show an anti-allodynic effect of AS-$Ca_V3.2$ in mononeuropathic rats, with a complete reversal of tactile allodynia. The shorter duration of this effect compared to the

antinociceptive/anti-hyperalgesic action of AS-$Ca_V3.2$ AS may suggest possible distinct $Ca_V3.2$ turnover or transcriptional control in the neuron subpopulations linked to either acute nociception or allodynia. Further studies would be needed to resolve this issue. Nonetheless, the overall effects of the AS-$Ca_V3.2$ AS corroborate with the high level of $Ca_V3.2$ transcripts in DRGs (Talley *et al*, 1999; Shin *et al*, 2003; Beedle *et al*, 2004) and the absence of T-type currents in DRG neurons from the $Ca_V3.2$ KO mice (Chen *et al*, 2003). The lack of $Ca_v3.1$ role is not surprising since $Ca_V3.1$ is not expressed in the sensory ganglia (Talley *et al*, 1999; Beedle *et al*, 2004) and the peripheral pain detection is normal in $Ca_V3.1$ KO mice (Kim *et al*, 2003). While expression of $Ca_V3.3$ mRNA is detected in DRGs, the functional properties of DRG-T-type currents are distinct from those of recombinant $Ca_V3.3$ channels. Therefore, the specialized function of $Ca_V3.2$ in peripheral pain pathways is consistent with the notion that the AS delivery and biological activity are restricted to DRGs.

The *in vitro* evaluation of the T-type channel knockdown supports the *in vivo* data. Our results demonstrate that AS treatment directed against $Ca_V3.2$ induces around 50% decline of $Ca_V3.2$ mRNA locally within the lumbar DRGs. These data also show that the AS-$Ca_V3.2$ treatment does not affect the very low mRNA levels observed for the other Ca_V3 genes, indicating no compensation for the loss of $Ca_V3.2$. The biochemical experiments first evidenced that our anti-$Ca_V3.2$ Ab specifically labelled the $Ca_V3.2$ proteins. When used on DRG membrane proteins of WT and $Ca_V3.2$ KO mice, a 230 kDa band near the predicted $Ca_V3.2$ full-length size disappeared in the samples from the KO animals. An additional aspecific immunoreactive lower band was also detected in both WT and KO animals. Importantly, when tested on proteins from treated rats at the end of the i.t. injection protocol, the anti-$Ca_V3.2$ Ab revealed that the AS treatment strongly reduced the $Ca_V3.2$ immunoreactivity specifically in the lumbar DRGs. These data correlate with both the high level of fluorescent-ODN uptake observed (Figure 1A–C) and the decrease of $Ca_V3.2$ transcripts in these tissues (Figure 1D). The functional exploration of the T-type channel activity on isolated lumbar DRG neurons further supported the AS treatment efficacy, as the T-type current density vanished away in small- and medium-sized neurons from AS-$Ca_V3.2$-treated rats, while no effect was noted on the HVA calcium currents. The slight difference between the lower inhibition of the $Ca_V3.2$ transcripts amount versus the protein level or the current density that we found was observed previously in other ODN-mediated channel or receptor knockdowns (Stone and Vulchanova, 2003). However, in comparison to these studies showing an ~40–70% reduction of the expression and/or activity of the targeted proteins, our results demonstrate a high level of AS effects and provide compelling evidences to support the behavioural results.

The higher efficacy of both ODNs targeting $Ca_V3.2$ (AS-Ca_V3-com and AS-$Ca_V3.2$) in the CCI model than in healthy rats, attested by the higher values of area under the time course curves of their effect, argues for an active pathophysiological role of $Ca_V3.2$. Modification of expression or redistribution of a number of primary afferents cellular markers has been reported following inflammation or neuropathy (Scholz and Woolf, 2002). There is little information regarding the plastic changes of voltage-gated calcium channels, although it was recently shown that $Ca_V2.2$ are upregulated following the induction of inflammation in carrageenan-injected rats (Yokoyama *et al*, 2003). In models of neuropathies, functional downregulation of HVA Ca^{2+} channels was generally reported in DRG neurons. Concerning T-type channels in medium-sized isolated DRG cell soma, either no modification or downregulation of their current density has been reported following axotomy or CCI (Baccei and Kocsis, 2000; Hogan *et al*, 2000; Andre *et al*, 2003; McCallum *et al*, 2003). These results seem to be in apparent contradiction with our data showing an enhanced pronociceptive role of T-type channels. However, it is important to note that all these studies describe T-type currents in the soma of isolated DRGs. Indeed, other pronociceptive channels have been shown to be unaffected or downregulated in the soma but redistributed and upregulated in peripheral nerve endings or in the central terminals in the dorsal horn of the spinal cord. They include TTX-resistant sodium channels ($Na_V1.8$ and $Na_V1.9$) or the capsaisin receptor (TRPV1) shown to be redistributed at the peripheral nerve endings during neuropathic or inflammatory conditions (Ji *et al*, 2002; Gold *et al*, 2003), or the calcium channel auxiliary $\alpha_2\delta_1$ subunit upregulated at the presynaptic terminal during neuropathy (Li *et al*, 2004). Since functional studies suggested a localization of T-type channels in both peripheral (Todorovic *et al*, 2001) and central (Bao *et al*, 1998) sensory nerve terminals, redistribution and possibly upregulation of $Ca_V3.2$ in distal sites would be consistent both with our AS data *in vivo* and with the previous data showing unaffected or reduced somatic currents during neuropathy. Interestingly, the $\alpha_2\delta_1$ subunit, containing the receptor site for the drug gabapentin effective against neuropathic pain, was shown to enhance recombinant $Ca_V3.2$ T-type calcium expression (Dolphin *et al*, 1999; Dubel *et al*, 2004). As $\alpha_2\delta_1$ is upregulated and distally redistributed during neuropathy, a consequential effect on $Ca_V3.2$ is an attractive hypothesis to explain the enhanced AS effects in CCI animals. In addition to modified expression levels, changes in channel functional activity can contribute to the pathological excitability, as reported for TTX-resistant sodium channels or acid-sensing ion channels (Gold *et al*, 1998; Voilley *et al*, 2001). In this context, acute application of reducing agents such as L-cysteine has been shown to augment T-type channel activity in nociceptive DRG neurons. As these agents can locally accumulate in inflamed or injured tissues, they may be involved in nociception via their action on T-type channels (Todorovic *et al*, 2001). Further experiments would be required to elucidate this issue.

Finally, the role of $Ca_V3.2$ in the distinct subpopulations of small and medium neurons remains to be clarified. Interestingly, in a subset of medium neurons identified as D-hair cells (Shin *et al*, 2003; Dubreuil *et al*, 2004), the high T-type channel density promotes membrane depolarization by controlling afterdepolarizations (ADPs). Similarly, in hippocampal neurons from epileptic rats, upregulation of '$Ca_V3.2$ like' T-type channels modifies neuronal firing with increased bursting and ADP (Su *et al*, 2002). Conversely, in some other medium DRG neurons (McCallum *et al*, 2003), coupling of T-type channels with Ca^{2+}-sensitive potassium channels may hamper bursting by generating after-hyperpolarizations, as for some midbrain neurons (Wolfart and Roeper, 2002). Nonetheless, at this stage, further exploration of the subcellular distribution, expression, and functional

activity of $Ca_V3.2$ will be needed to elucidate fully the molecular mechanisms underlying the T-type channel pronociceptive role in naïve, neuropathic, and inflammatory conditions.

In conclusion, the present study shows for the first time that $Ca_V3.2$ AS treatment leads to an effective and functional knockdown of T-type channels in primary afferent damage-sensing neurons. This $Ca_V3.2$ gene silencing provides a clear demonstration of the specialized function of this channel in nociception in both acute and neuropathic conditions. Compared to other channels or receptor knockdowns using intrathecal AS ODN, we report markedly robust pain-relief effects without any sign of behavioural toxicity or motor dysfunction. Given the prominent expression of $Ca_V3.2$ to sensory neurons in the peripheral nervous system, selective pharmacological antagonists targeting these channels are likely to mediate a potent analgesia with few side effects. In this respect, the discovery of selective T-type channel antagonists might be of great interest for the clinical treatment of pain.

Materials and methods

Oligodeoxynucleotides targeting Ca_V3 subunits

AS phosphorodiester ODNs were designed based on rat Ca_V3 sequences (McRory *et al*, 2001; GenBank nos. AF290212, AF290213, AF290214) in regions lacking known splice variants. They were synthesized by Sigma-Genosys and sequences were as follows: AS-Ca_V3-com, TCCACCACCACGCCCACAAACATGTT; AS-$Ca_V3.1$, CGA GACCCATTGGCATCCCT; AS-$Ca_V3.2$, CCACCTTCTTACGCCAGCGG; AS-$Ca_V3.3$, GCTGAGGGCGGCTTGTGTTT. Two ODNs with scramble arrangement in the base composition compared to the 26-mer common and 20-mer specific ASs were used as controls for sequence-independent effects of ODN treatments. Their sequences were as follows: Mismatch-1, TCACCCAGCACCCCCAACACATAGTT; Mismatch-2, TACTGTACTTGCGAGGCCAC. A blast search revealed that these mismatch ODNs were not complementary to any registered nucleotide sequences. To visualize the ODN uptake, the 5′ ends of AS-Ca_V3-com, AS-$Ca_V3.2$, and Mismatch-1 were coupled to a fluorescein group. ODNs were reconstituted in saline before administration.

Intrathecal ODN administration

I.t. administrations of ODNs (12.5 μg/rat) or saline were performed in a volume of 10 μl via direct transcutaneous injection (with a 25-gauge needle connected to a 25 μl Hamilton syringe) between the L5 and L6 dorsal spinous processes (Mestre *et al*, 1994) under animal anaesthesia with isofluran (3.5%). This treatment was repeated twice daily for 4 days (days 1–4). This protocol was based on previous studies demonstrating an efficient knockdown of sodium channels in sensory neurons *in vivo* (Lai *et al*, 2000). T-type calcium channel turnover has been estimated to be 3 days in primary culture of sensory neurons (Lambert *et al*, 1998); therefore, a 4-day AS treatment was applied followed by exploration of the behaviour of animals. Pain scores were determined using standard methods in strict conformity with ethical standards (Zimmermann, 1983; see Supplementary data) before ODN treatments and then on day 4 in the afternoon, and on days 5, 6, 8, 9, 10, 11, and 12, at the same time. In each experiment, 5–7 animals per group were used. Treatments were randomized and all experiments were performed blind by the same experimenter using the method of equal blocks to avoid any uncontrollable environmental influence that might induce a modification in behavioural response.

QRT–PCR analysis

Total RNA was prepared using the Micro-to-Midi purification system (Invitrogen) and treated with DNaseI (Ambion). cDNA was synthesized using random primers and the Superscript first-strand synthesis system (Invitrogen). Additional reactions were performed in the absence of reverse transcriptase to assess contamination by genomic DNA. Real-time PCR analysis was performed on an Applied Biosystems instrument (ABI Prism 7000). Primers were designed for each Ca_V3 gene and for two housekeeping genes (HPRT and synaptophysin) to generate amplicon sizes of 67–78 bp (see Supplementary data). The PCR reaction was performed using 7.5 μl of TaqMan PCR Master Mix and 2.5 μl of cDNA template. Average C_t values from triplicate PCR reactions were normalized to average C_t values for reference gene from the same cDNA preparation.

Western blotting

Proteins were made as previously described (Djouhri *et al*, 2003), and their concentration was determined using a BCA assay (Pierce). DRGs and spinal cords of six WT and six $Ca_V3.2$ KO mice were collected. Lumbar and thoracic DRGs and spinal cords of treated rats were collected in two independent experiments using groups of five animals per condition. Proteins were separated by SDS–PAGE on 4–20% gradient gels (Bio-Rad), and transferred onto nitrocellulose membranes. Membranes were blocked with 5% powdered nonfat milk. $Ca_V3.2$ protein was detected with an anti-$Ca_V3.2$ affinity-purified rabbit polyclonal Ab at a 1:5000 dilution (specificity characterized by TP Snutch and JE McRory). The $Ca_v3.2$ Ab epitope corresponds to amino acids 1193–1274 of the $Ca_v3.2$ carboxyl tail (accession no. AF290213). HRP-conjugated secondary anti-rabbit Ab (Amersham) was used at 1:5000 dilution. The signal was detected using the SuperSignal West Pico Chemiluminescent system (Pierce). The membranes were stripped and reprobed with an anti-β-tubulin (1:1000; Sigma) or an anti-Erk2 (1:1000; Cell Signalling Tech.). Relative intensity of the $Ca_V3.2$ immunoreactivity compared to the β-tubulin control was evaluated on scanned images of the blots.

Electrophysiological recordings

At the end of the ODN treatment, dissociated rat L3–L6 DRG cells were prepared as previously described (Beedle *et al*, 2004) and recordings were completed within 16 h of plating. Whole-cell patch-clamp recordings were made at room temperature from small and medium DRGs ($\leqslant$40 μm diameter) with an Axopatch 200A amplifier controlled by the pClamp software (Axon Instruments). The bath solution contained (in mM) 2 $CaCl_2$, 160 TEACl, 10 glucose, and 10 HEPES (pH 7.4 with TEAOH). Pipettes of resistance of 1–2 MΩ were filled with an internal solution containing (in mM) 110 CsCl, 3 $MgCl_2$, 10 EGTA, 10 HEPES, 3 Mg-ATP, and 0.6 GTP (pH 7.4 with CsOH). Analysis was performed with Pclamp9, Excel, and GraphPad Prism software. Results are presented as the mean $\pm$ s.e.m., and compared using Student's *t*-tests.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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