Antinociceptive effect of antisense oligonucleotides against the vanilloid receptor VR1/TRPV1

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Abstract

To examine the role of the vanilloid receptor TRPV1 in neuropathic pain, we assessed the effects of the receptor antagonist thioxo-BCTC and antisense oligonucleotides against the TRPV1 mRNA in a rat model of spinal nerve ligation. In order to identify accessible sites on the mRNA of TRPV1, the RNase H assay was used, leading to the successful identification of binding sites for antisense oligonucleotides. Cotransfection studies using Cos-7 cells were employed to identify the most effective antisense oligonucleotide efficiently inhibiting the expression of a fusion protein consisting of TRPV1 and the green fluorescent protein in a specific and concentration-dependent manner. In an \textit{in vivo} rat model of spinal nerve ligation, intravenous application of the TRPV1 antagonist thioxo-BCTC reduced mechanical hypersensitivity yielding an ED\textsubscript{50} value of 10.6 mg/kg. Intrathecal administration of the antisense oligonucleotide against TRPV1, but not the mismatch oligonucleotide or a vehicle control, reduced mechanical hypersensitivity in rats with spinal nerve ligation in a similar manner. Immunohistochemical analysis revealed neuropathy- and antisense-associated regulation of TRPV1 protein expression in spinal cord and dorsal root ganglia. Our data demonstrate comparative analgesic effects of a TRPV1 antagonist and a rationally designed TRPV1 antisense oligonucleotide in a spinal nerve ligation model of neuropathic pain and thus, lend support to the validation of TRPV1 as a promising target for the treatment of neuropathic pain.

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1. Introductory statement

The capsaicin or vanilloid receptor VR1 is a member of the transient receptor potential (TRP) family and is thus also referred to as TRPV1 (Montell et al., 2002). It is predominantly expressed by nociceptive sensory neurons and functions as a cation channel (Caterina et al., 1997; Tominaga et al., 1998). TRPV1 is activated by a wide array of pain-producing stimuli including capsaicin, noxious heat and low pH (for reviews, see Cortright and Szallasi, 2004; Tominaga and Tominaga, 2005) and is furthermore sensitized by pain-associated intracellular signaling pathways (e.g. protein kinase A, protein kinase C, ATP, phosphatidylinositol diphosphate (PIP\textsubscript{2})) in a way that makes it a potentially pivotal regulator of nociceptor activity (Benham et al., 2002; Di Marzo et al., 2002). TRPV1 is therefore regarded as a promising target for the development of new analgesic strategies. The receptor agonist capsaicin is already being used for treatment of neuropathic pain, but its initial pungency hinders its broad application (The Capsaicin Study Group, 1991).

The crucial involvement of TRPV1 to drive inflammatory pain is well established by analysis of TRPV1 knock-out mice (Caterina et al., 2000; Davis et al., 2000) as well as by investigations employing TRPV1 antagonists (Garcia-Martinez et al., 2002; Walker et al., 2003). Chemical ablation of TRPV1...
expressing capsaicin sensitive neurons with the capsaicin agonist resiniferatoxin (RTX) also proved that TRPV1 mediates inflammatory pain including cancer pain (Karai et al., 2004). In contrast, the role of TRPV1 during neuropathic pain is less clear. After peripheral nerve injury a distinct regulation of TRPV1 expression was observed (Hudson et al., 2001; Fukuoka et al., 2002; Kanai et al., 2005). Intrathecal application of the TRPV1 antagonist capsazepine blocks αδ-fiber evoked responses in dorsal horn neurons of rats after sciatic nerve ligation (SNL) (Kelly and Chapman, 2002). In contrast, the analysis of TRPV1 null mice versus wild-type mice revealed no difference in a model of partial SNL (Caterina et al., 2000). Further studies using capsazepine indicated possible species differences. Capsazepine reversed mechanical hyperalgesia in a guinea pig model of partial SNL, but had no effect in rat or mouse models of neuropathic pain (Walker et al., 2003).

In recent years, a number of other TRPV1 antagonists have been developed. These include halogenated vanilloid compounds like iodo-resiniferatoxin (RTX) and 6-iodo-nordihydrocapsaicin (Wahl et al., 2001; Appendino et al., 2003; Karai et al., 2004), arginine-rich peptides and peptidoids (Planells-Caçador et al., 2000; García-Martínez et al., 2002; Himmel et al., 2002), two series of thiourea compounds (Lee et al., 2001; Wang et al., 2002), and carbonamides such as BCTC (N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide) or SB-367691 (Pomonis et al., 2003; Valenzano et al., 2003; Gunthorpe et al., 2004). With the exception of BCTC, data regarding the efficacy of these TRPV1 antagonists in animal models of neuropathic pain are lacking, and hence the role of TRPV1 in neuropathic pain is still under debate (Pomonis et al., 2003; Kanai et al., 2005).

A very elegant way to study the pain-relevance of genes expressed in DRGs was introduced by Porreca and coworkers who demonstrated that repeated intrathecal injection of unmodified antisense oligodeoxyribonucleotides (AS ODNs) results in a specific and reversible knock-down of the target protein that allows to analyse changes in nociceptive behavior (Bilsky et al., 1996; Porreca et al., 1999; Joshi et al., 2000; Dogrul et al., 2002; Lai et al., 2002; Parada et al., 2003). These studies as well as a number of further investigations have demonstrated the potential of antisense strategies for pain research (for reviews, see Stone and Vulchanova, 2003; Kurreck, 2004).

Accordingly, we re-examined the role of TRPV1 during neuropathic pain by a comparative analysis of thioxo-BCTC and TRPV1 AS ODNs in SNL rats. Both strategies revealed that impairment of TRPV1 results in a reduced mechanical hypersensitivity in the animal model under investigation lending further support to the concept that TRPV1 is causally involved in neuropathic pain. As the TRPV1 antisense oligonucleotide was found to be as efficacious as the TRPV1 antagonist we propose that TRPV1 antisense oligonucleotides are useful to validate TRPV1 as drug target in different states of pain. If low molecular weight antagonists are not available antisense oligonucleotides can be used in general to validate other presumed drug targets.

2. Materials and methods

2.1. Oligonucleotides

Unmodified DNA oligonucleotides as well as phosphorothioates for in vitro and cell culture experiments were obtained from MWG Biotech AG (Ebersberg, Germany). Oligonucleotides for in vivo applications were purchased from IBA GmbH (Goettingen, Germany). The sequences of oligonucleotides V15 and V15MM were:

V15: CAT GTC ATG AGC GTT AGG
V15MM: CAT GCT ATG AGC GTT GAG.

2.2. Cell culture and transfection

Cos-7 cells (African green monkey kidney fibroblasts) were grown in a humidified atmosphere at 37 °C with 5% CO₂ in Dulbecco's modified Eagle medium (PAA laboratories, Coelbe, Germany), supplemented with 10% FCS (PAA laboratories), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Invitrogen, Karlsruhe, Germany). Exponential cell growth was maintained by passage of the cells before they reached confluency.

The day before transfection, cells were resuspended in medium without antibiotics and transferred to 24-well plates at a density of 8 x 10⁴ cells per well. Transfection and cotransfection experiments with a rat TRPV1-containing plasmid (pcDNA.3/CT-GFP-TOPO (Invitrogen)) and the respective amounts of the phosphorothioate antisense oligonucleotides V15 or V15MM were done with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cells were incubated at 37 °C for at least 24 h post-transfection.

CHO K1 cells were routinely grown as monolayers in F-12 nutrient mixture (Invitrogen) supplemented with 1-proline (18 µg/ml), 10% fetal bovine serum and maintained under 5% CO₂ at 37 °C. The selected stable CHO K1 cell clones expressing the rat and human TRPV1, rTRPV1-CHO and hTRPV1-CHO, respectively, were grown in medium containing 100 µg/ml G-418 (Invitrogen).

2.3. Fluorescence microscopy and immunoblotting

For fluorescence microscopy, the medium was aspirated from the cells and 200 µl phosphate-buffered saline (PBS) were added. Fluorescence images were taken directly from living cells using a Leica DM IRB fluorescence microscope.

For Western blot experiments, cells were lysed directly in 24-well plates with lysis buffer (125 mM Tris–HCl, pH 6.8; 4% SDS; 1.4 M 8-Mercaptoethanol; 25% glycerol and 0.05% bromophenol blue). The lysate was boiled for 5 min at 95 °C and equal amounts of protein were separated on 10% polyacrylamide gels. Transfer of separated proteins to PVDF membranes (GE Healthcare/Amersham, Freiburg, Germany) was performed with a semi-dry blotter (BioRad, Munich, Germany). Immunostaining was done with a GIP-antiserum (Invitrogen, Germany) that was diluted 1:5000 in Blotto. Secondary antibodies were conjugated with alkaline-phosphatase (AP) (Chemicon, Temecula, CA, USA) and diluted 1:5000. CDP-Star (Roche, Mannheim, Germany) was used as a chemiluminescence substrate for AP. Membranes were reprobed with a monoclonal mouse antibody against actin (Chemicon).

2.4. Northern blot experiments

A digoxigenin (Dig)-labeled antisense RNA was prepared from the plasmid pCR II TOPO that contained the TRPV1 cDNA. The plasmid was linearized with Smal followed by an in vitro transcription with Dig-labeled nucleotides (Roche), resulting in a 1.7 kb RNA probe. Total RNA was prepared from transfected Cos-7 cells (RNasey; Qiagen, Hilden, Germany) and separated on 1.2% formaldehyde gels. Northern blotting was performed with the Nord-ermMax kit (Ambion, Austin, TX, USA) according to the manufacturer’s recommendations. Hybridization and washing steps were performed at 68 °C. Detection of the hybridized probe was done with the digoxigenin-detection system (Roche) using a digoxigenin-specific antibody, which was conjugated with AP. CDP-Star was used as a substrate for AP and chemiluminescence was detected with X-ray films.
2.5. Measurement of intracellular calcium levels using the FLIPR™

rTRPV1- and hTRPV1-CHO cells were seeded into black-walled clear-base poly-D-lysine coated 96-well plates (Becton Dickinson, Meylan Cedex, France) at a density of 25,000 cells per well in F-12 medium, supplemented as described above, and cultured overnight. The cells were then incubated with Hank’s balanced salt solution supplemented (Invitrogen) with 2.5 mM probenecid and 20 mM Hepes (all Sigma, Taufkirchen, Germany), containing the cytoplasmic calcium indicator Fluo-4AM (4 μM; Molecular Probes, Eugene, OR, USA) at 37 °C for 30 min. The cells were washed twice with Hank’s balanced salt solution supplemented with 2.5 mM probenecid and 20 mM Hepes, resuspended in the same buffer, and incubated for 15 min at 37 °C. Subsequently, the plates were inserted into a fluorometric imaging plate reader (FLIPR™; Molecular Devices, Sunnyvale, CA, USA), and the fluorescence (λex = 488 nM, λem = 510–570 nM) from intracellular calcium ([Ca2+]i) was determined before and after the addition of various concentrations of test compounds (Sullivan et al., 1999; Jerman et al., 2000). To test for a possible antagonistic activity of the drugs, capsaicin (50 μM/well, 100 mM final concentration) was added to each well containing TRPV1-transfected cells after 6 min.

2.6. Animal model of neuropathic pain

Adult male Sprague Dawley rats (170–310 g, obtained from Janvier, Le Genest Saint Isle, France) were used in the study. Animals were kept under standard laboratory conditions with free access to standard laboratory food and tap water. The experiments were performed in accordance with ECC guidelines (86/609/EEC) for the use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were tested in randomized groups of 10 for each dose and vehicle controls. Although the operators performing behavioral tests were not formally ‘blinded’ with respect to the treatment, they were not aware of the nature of the differences between the drugs and the study hypothesis. Spinal nerve ligation of the left L5/L6 spinal nerves was performed in 80 male Sprague Dawley rats according to Kim and Chung (1992). For antisense treatment spinal catheters were implanted into 50 rats at the same time according to Pogatzki et al. (2000). Four to 6 days after operation mechanical baseline thresholds were taken on the ipsi- and contralateral hindpaw by means of an electronic von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA, USA). Proper placement of the spinal catheters was confirmed by lidocaine application (10 μl, 2% resulting in transient bilateral hind limb paralysis. Animals that failed within this lidocaine-test were not included in the subsequent behavioral tests. After baseline testing 45 μg of the AS ODN against TRPV1 (V15, n = 10) or a mismatch control oligonucleotide (V15MM, n = 10) in 0.9% NaCl or vehicle (0.9% NaCl, n = 10) was applied once on the first day and b.i.d. on the following 3 days. Mechanical withdrawal thresholds were tested 30 min after the first daily oligonucleotide administration. Results are presented as % maximal possible effect (% MPE) on the ipsilateral side taking the baseline as 0% and the withdrawal threshold of a sham control group as 100% MPE.

Thioxo-BCTC was synthesized at Grünenthal GmbH. The substance was dissolved in 5% DMSO, 75% PEG400 and 20% saline and tested after i.v. administration in rats 1–3 weeks after SNL. Thirty animals were tested more than once and underwent a washout period of at least 7 days. Withdrawal thresholds were measured on the ipsilateral paw 0.5 h before and 0.5, 1, and 3 h after administration by means of an electronic von Frey anesthesiometer (Somedic AB, Malmö, Sweden). Data were analysed by means of repeated measures ANOVA. Significance of treatment effect, time effect or treatment × time interaction were analysed by means of Wilks’ Lambda statistics. In case of a significant treatment effect, pair wise comparison was performed by Fisher’s LSD test. Results were considered statistically significant at p < 0.05. ED50 values and 95% confidence intervals (95% CI) were determined at the time of the peak effect by semi-logarithmic regression analysis or according to Litchfield and Wilcoxon (1949).

2.7. Open field motility test in rats

The possible sedative and muscle relaxant actions of compounds were evaluated in the open field test (MotiSystem, TSE, Bad Homburg, Germany).

Thirty-two infrared emitters and opposed sensors were evenly distributed on the length of the X- and Y-axis of the test field (45 cm x 45 cm). After the administration of the test compound, the rat was placed individually into the centre of the open field and the activity was measured for 5 min. The locomotor activity was quantified as the number of light beam interruptions and the overall movement distance (m) over this period was calculated. Group size was 10 animals. Control groups received vehicle injection.

2.8. Immunohistochemistry

The spinal cords were removed after behavioral testing, dissected and immersed overnight in Bouin Hollande fixative. After fixation the tissue was extensively washed in 70% 2-propanol and processed for routine paraffin embedding. Polyclonal antibodies were raised in rabbits against the C-terminus of rat TRPV1 (CFKDSMVCPEK) and used at 1:2000 dilution. Sections of 7 μm thickness were mounted on adhesive slides. After deparaffinization and blocking of endogenous peroxidase with 0.1% perhydrol in methanol at RT for 30 min, sections were heated at 92–95 °C for 15 min in 0.01 M citrate buffer, pH 6.0. After incubation for 30 min in 50 mM PBS containing 5% bovine serum albumin, nonspecific binding of avidin−biotin was blocked by the 30% Biotin/ Avidin Blocking Kit (Vector Laboratories, Burlingame, CA, USA) at RT for 20 min. Sections were incubated with the primary antibodies overnight at 16 °C followed by 2 h at 37 °C. For negative controls, primary antibodies were omitted. After several washes in distilled water followed by rinsing in 50 mM PBS, species-specific biotinylated secondary antibodies (Dianova, Hamburg, Germany) were applied for 45 min at 37 °C. After another series of washes, sections were incubated for 30 min with the ABC reagents (Vectastain ABC-Kit; Vector Laboratories, Burlingame, CA, USA) followed by a nickel-enhanced diaminobenzidine reaction (0.125 μg/ml diaminobenzidine and 0.75 μg/ml ammonium nickel sulfate) for 10 min at room temperature.

2.9. Evaluation of immunohistochemical data

Sections were photographed with an Olympus AX70 microscope under constant light and camera conditions. Semiquantitative immunocytochemistry (ICC) analysis was conducted using the MCID M4 image analysis system (Imaging Res., St. Catharines, Canada). For spinal cord sections data are presented as total target area per μm². For DRG the cells were counted and data are presented as number of cells per 50,000 μm². The results are presented as mean ± S.E.M. from five animals per group. p < 0.05 and ***p < 0.001 indicate a significant difference between ipsilateral and contralateral DRG and sides of the spinal cord. For statistical analysis the one-way analysis of variance ANOVA Bonferroni test was used.

3. Results

To investigate the functional role of the vanilloid receptor TRPV1 in neuropathic pain processes in more detail, the analgesic effects of a low molecular weight receptor antagonist and an AS ODN directed against the TRPV1 mRNA were evaluated in vivo and in vitro. The plasticity of TRPV1 expression after the neuropathic lesion and under TRPV1 antisense treatment was monitored by immunocytochemistry.

3.1. Thioxo-BCTC is a potent TRPV1 antagonist

BCTC has been shown to be a potent antagonist of TRPV1 (Valenzano et al., 2003). Here, we wanted to investigate the properties of thioxo-BCTC, which displays higher solubility. We found this derivative to inhibit capsaicin induced Ca²⁺ release in CHO-K1 cells recombinantly expressing rat or human TRPV1 receptor, as measured by FLIPR experiments.
results are presented as % of possible maximum effect (MPE). Data are means ± S.E.M. (n = 10 rats/group; * p < 0.05). (B) Thioxo-BCTC shows no effect on locomotor activity in the open field assay in rats. Rats received intraveneous administration of 0 and 21.5 mg/kg thioxo-BCTC and were tested also in the rat open field model. Intravenous administration of 21.5 mg/kg thioxo-BCTC, i.e. the highest dose tested in SNL rats, had no effect on locomotor activity (Fig. 1B). After having shown that the TRPV1 antagonist thioxo-BCTC reduces mechanical hypersensitivity in an animal model for neuropathic pain, we aimed at developing efficient AS ODNs to prevent the synthesis of the TRPV1 protein.

3.3. Identification of an efficient antisense oligonucleotide against TRPV1

In a previous study, we screened 32 AS ODNs against the mRNA of TRPV1 in a messenger walk screening in the presence of RNase H in vitro (Kurreck et al., 2002a). The AS ODNs were 18 nucleotides long and targeted all GUC triplets in the translated region, which can be used as cleavage sites for hammerhead ribozymes. Two of the AS ODNs, named V15 and V29, were found to induce almost complete degradation of the TRPV1 transcript by RNase H. Since V15 showed a higher inhibitory activity in cell culture than V29 (Grünweller et al., 2003), this AS ODN was used for all further experiments. We first wanted to characterize the antisense activity of V15 in cell culture. To this end, we generated a plasmid encoding a fusion protein of TRPV1 and the green fluorescent protein (GFP). Transfection of Cos-7 cells with this plasmid resulted in a green fluorescent phenotype that can easily be monitored in living cells by fluorescence microscopy (Fig. 2A).

We then performed cotransfection experiments with the antisense phosphorothioate V15 or the mismatch control oligonucleotide V15MM and observed a specific and concentration-dependent inhibition of TRPV1-GFP expression between 50 and 100 nM: only few cells were still fluorescing after treatment with 50 nM V15 and no fluorescence was observed in the presence of 100 nM V15 (Fig. 2A). In contrast, cotransfection of V15 MM did not reduce the green fluorescence.

These findings were confirmed in a Western blot: the band representing TRPV1-GFP was significantly reduced after treatment with 50 nM V15 and was virtually absent in the sample that contained 100 nM V15 (Fig. 2B). Again, the mismatch control oligonucleotide V15MM had only minor effects. An anti-actin antibody was used to confirm equal loading of proteins in each lane.

Finally, the antisense effect was confirmed at the RNA level. Equal amounts of RNA prepared from cells transfected with TRPV1-GFP, TRPV1-GFP and 100 nM V15, or TRPV1-GFP and 100 nM V15MM were loaded onto the gel. As can be seen in the Northern blot using a TRPV1-specific probe no TRPV1-GFP mRNA could be detected after treatment with 100 nM V15, whereas no degradation was observed with the control
Actin serves as a loading control. We therefore conclude that V15 is a suitable oligonucleotide to efficiently suppress TRPV1 expression in cell culture. The antisense effect was not only demonstrated at the protein level, but at the mRNA level as well, indicating degradation of the target mRNA by RNase H. The mismatch oligonucleotide with inversions of every fifth and sixth nucleotide had only minor effects and can therefore serve as a control for the specificity of the antisense effect.

3.4. Uptake of AS ODNs in cells of the lumbar DRG

We next wanted to investigate the pain-modulating effects of TRPV1 in an animal model for neuropathic pain. In order to prove the delivery of the oligonucleotides into the DRG, the TRPV1 AS ODNs were covalently labeled with the fluorescent dye Cy3. For the uptake studies the Cy3-labeled ODN was delivered as a single bolus (45 µg, i.th., n = 5) or twice with a second bolus 8 h later (n = 5). Spinal cord and DRGs from the animals were recovered 24 h after the first injection. Fluorescence microscopy revealed strong fluorescence in DRG cell bodies, with no apparent differences between the two application groups (Fig. 3). The fact that not all cells contain fluorescence but most of them appear to be neuronal cell bodies makes it unlikely that unspecific diffusion or dissection artifacts are responsible for the observed fluorescence. The fluorescence was primarily confined to the cytoplasm of the cells, where it was of evenly distribution.

3.5. Changes in TRPV1 expression after spinal nerve ligation

The distribution of TRPV1-immunoreactivity (IR) in lumbar spinal cord and DRGs in normal and nerve-injured rats was assessed using immunohistochemistry in conjunction with confocal microscopy. As expected the TRPV1-IR in spinal cord was observed in the substantia gelatinosa. Interestingly, the comparative analysis of spinal cord segments L3–L6 showed the highest TRPV1-IR in L6 which gradually decreased up to...
Fig. 4. Changes of TRPV1 protein expression in lumbar (L3–L6) spinal cord after SNL. (A) Series of bright field photomicrographs depicting TRPV1-immunoreactivity in the dorsal spinal cord 10 days after L5/L6 SNL at different lumbar levels (panel A). Note differences in the TRPV1 staining pattern in the superficial dorsal horn depending on the spinal cord level. The highest immunostaining was observed at L6, the lowest at L3. The spinal nerve ligation leads to a dramatic reduction of TRPV1-immunoreactivity on the ipsilateral side (arrows) as compared to the contralateral side. Scale bar denotes 100 μm. (B) High power bright field micrographs of the superficial dorsal horn and dorsal roots of the L6 level from (A). Note regional accumulation and partial superimposition of strongly TRPV1 positive fibers besides less strongly stained TRPV1 fibers and reduction of TRPV1 immunoreactive fibers and terminals in the ipsilateral superficial dorsal horn (upper panel). There are numerous small diameter TRPV1 positive cross profiles in a cross-sectioned part of the contralateral dorsal root whereas TRPV1 positive fiber profiles are virtually absent in the ipsilateral dorsal root (lower panel). Scale bars denote 25 μm. (C) Semi-quantitative analysis of TRPV1 immunostaining intensities in lumbar L3–L6 spinal cord. Note the decline of the TRPV1 signal in the dorsal spinal cord ipsilateral to the injury and the differences in TRPV1 immunostaining on the contralateral side depending on the lumbar level.
L3 (Fig. 4C). After SNL a significant ipsilateral decrease of TRPV1 protein in lumbar spinal dorsal horn at the levels L5 and L6 was noted (see arrows in Fig. 4A, right panel; Fig. 4C), whereas in L3 and L4 no significant ipsilateral changes could be observed (Fig. 4A, left panel; Fig. 4C).

Higher magnification of the L6 dorsal horn revealed that both densely packed incoming fibers with strong VR1 immunoreactivity and segregated fibres less strongly staining for VR1 were reduced in the ipsilateral superficial dorsal horn (Fig. 4B, upper panel). High power micrographs of L6 dorsal roots demonstrate a drastic reduction of TRPV1 protein in the ipsilateral L6 dorsal root as compared to the contralateral side (Fig. 4B, lower panel). Here, TRPV1 fibers were virtually absent in the ipsilateral dorsal root while clearly distinguishable small diameter cross profiles were seen in the contralateral dorsal root (Fig. 4B, lower panel).

In lesioned DRGs, TRPV1-IR protein was observed in the small and medium diameter neurons. Ten days after SNL we...
found a nearly complete loss of the TRPV1 expression in the ipsilateral L5 (and L6) DRGs as compared with the contralateral DRGs. This is shown for the DRGs of L5 segment (Fig. 5, lower panel). No significant changes of TRPV1-IR were detected in the ipsilateral L4 DRG (Fig. 5, upper panel) as compared to the contralateral side.

3.6. Changes in TRPV1 expression after ODN treatment of SNL rats

The effect of TRPV1 AS ODN treatment on the TRPV1 expression was assessed by quantification of TRPV1-IR in DRGs and spinal cord. Analysis of DRG and spinal cord tissue 5 days after continuous intrathecal ODN treatment showed no differences between the antisense and the mismatch ODN-treated SNL rats (Fig. 6).

3.7. TRPV1 antisense oligonucleotides reduce mechanical hypersensitivity associated with nerve injury

Intrathecal administration of AS ODNs was demonstrated to be a suitable technique to investigate the role of pain receptors expressed in the DRGs (Porreca et al., 1999; Lai et al., 2002). Therefore, catheters were implanted at the same time as SNL and the correct positioning of the catheters was tested by injecting a small dose of lidocaine (see Section 2). The intrathecal administration of the TRPV1 AS ODNs in rats with SNL partially reversed the mechanical hypersensitivity beginning at the second day after treatment (Fig. 7). The anti-nociceptive effect further increased reaching a significant effect between days 4 and 5 (repeated measures ANOVA outcome: treatment \( F(3, 36) = 96.681, p < 0.0001 \); time \( F(4, 33) = 5.030, p = 0.003 \); treatment \times time \( F(12, 87) = 2.026, p = 0.031 \)). The Cy-3 labeled oligonucleotide used to demonstrate uptake of the AS ODNs into the DRG as described above led to a similar reduction of the mechanical hypersensitivity as the unlabeled AS ODN (data not shown). In contrast, no effect could be observed using the mismatch oligonucleotide (MM) (Fig. 7). The fact that TRPV1 AS ODN or MM ODN showed no effects on the contralateral paws of the SNL rats underlines the specificity of the TRPV1 antisense effect on the ipsilateral side. No side effects in the animals, which gained weight and behaved normally, could be observed during the whole duration of treatment with either AS ODN or MM ODN.

4. Discussion

The aim of the present study was to investigate the involvement of TRPV1 in neuropathic pain of rats subjected to SNL. Two complementary approaches have been employed to this end: we used a low molecular weight compound that acts as a receptor antagonist as well as AS ODNs against the TRPV1 mRNA. The AS ODN has previously been shown to induce RNase H mediated cleavage of TRPV1 mRNA in vitro (Kurreck et al., 2002a) and to silence TRPV1-GFP expression in cell culture (Grünweller et al., 2003). Frank Porreca and co-workers established a procedure, according to which twice daily intrathecal administration of AS ODNs produces a selective block of protein expression in spinal cord, DRGs or nerve terminals (e.g. Porreca et al., 1999; Lai et al., 2002; Gold et al., 2003). The experimental design of the antisense treatment presented here is adapted according to this standard procedure. As expected, we were able to detect fluorescently labeled oligonucleotides in DRG neurons; no side effects were observed after antisense treatment; a mismatch ODN did not exert effects on the mechanical hypersensitivity in SNL rats and the time course for the onset of the antisense effect was around 2 days.

Studies from recent years reveal a complex pattern for the regulation of TRPV1 expression in different lumbar segments. Our observations are in good agreement with the findings of Hwang and Valschanoff (2003) who also reported a segmental TRPV1 expression gradient with highest expression in the L6 segment of spinal cord. Immunohistochemical analysis of TRPV1 expression in SNL rats showed a nearly complete downregulation of TRPV1 expression in SNL rats. A somewhat different finding was reported by Kanai et al. (2005), who observed a moderate increase of TRPV1 protein in the ipsilateral dorsal horn after chronic constriction injury as determined by Western blot. Differences in TRPV1 regulation can be explained by differences in the nerve lesion models used: Bennett ligation is likely to affect more segments as compared to the strictly bisegmental L5/L6 lesion model of the spinal nerve used in the present study. On the other hand, the dramatic dropout of TRPV1 input from L5 and L6 may have masked an enhanced input of TRPV1 from supraspinal and infraspineral DRGs.

The exact site of action of the antisense oligonucleotides thus remains to be clarified in future studies. TRPV1-immunoreactivity was significantly reduced in the ipsilateral L5 and L6 segments after SNL. The antisense treatment might
further down regulate TRPV1 in DRG neurons at these levels resulting in reduction of pain. However, it was impossible to demonstrate further knockdown of TRPV1 expression by antisense treatment. Alternatively, and more likely, the antisense effect may be targeted against a subpopulation of TRPV1 neurons in supraspinal L4 DRGs which react with an upregulation of TRPV1. In fact, such an upregulation of TRPV1-IR in C-fibers and especially small diameter A-fibers in supraspinal L4 DRGs of SNL rats has already been described (Hudson et al., 2001; Fukuoka et al., 2002). However, the relatively small trend (this study) of SNL-induced increase of TRPV1-IR and of approx. 10–37% (Hudson et al., 2001; Fukuoka et al., 2002) in supraspinal L4 DRGs was too small for a quantitative evaluation of an antisense effect.

The observation that behavioral and functional changes do not always correlate with the extent of modulation of the target gene expression after antisense treatment was also pointed out in a review summarizing antisense studies in the field of pain research (Stone and Vulchanova, 2003). As an example the authors mentioned a study by Wahlestedt et al. (2000), in which a 35–55% reduction in deltorphin II binding was associated with 80–90% reduction in deltorphin II analgesia. This frequent discrepancy of in vivo effect and protein down regulation could be due to spare receptors or the fact that the in vivo effect and the expression analysis may measure different pools of the target protein. A third possible mechanism of TRPV1 antisense action is based on the results of Tohda et al. (2001), who demonstrated a bidirectional axonal transport of TRPV1 mRNA in primary afferent neurons after carrageenan inflammation. Therefore, the TRPV1 AS ODNs may also inhibit the translation of the TRPV1 mRNA that takes place in the peripheral endings of the primary afferent neurons. In any case, knockdown effects of the TRPV1 AS ODNs on neuropathic-induced TRPV1 protein levels in vivo were too subtle to be detectable.

In the last years, the potent TRPV1 antagonist BCTC was developed and characterized in vitro (Valenzano et al., 2003) and in vivo (Pomonis et al., 2003; Kanai et al., 2005). BCTC given p.o. or i.th. was shown to reduce mechanical hyperalgesia and mechanical allodynia 2 weeks after SNL. In order to improve the pharmacokinetic properties of the substance, we synthesized the derivative thioxo-BCTC. As observed for the AS ODN we found a significant analgesic effect of thioxo-BCTC when investigating mechanical hypersensitivity in rats after SNL. Interestingly, Hudson et al. (2001) showed that the increase of TRPV1-IR in L4 DRGs of SNL rats is not only seen in C-fibers but even to a higher extent in myelinated A-fibers. This could be an explanation for the ability of thioxo-BCTC and the TRPV1 AS ODNs to block mechanical hypersensitivity following nerve injury.

Taken together, our present study demonstrates by two independent approaches that TRPV1 is of functional relevance in neuropathic pain. The low molecular weight receptor antagonist thioxo-BCTC as well as the intrathecally injected AS ODN V15 reduced mechanical hypersensitivity in a rat model of SNL. These results indicate that TRPV1 is a suitable target for new analgesics to treat neuropathic pain. We consider the strategy described here to employ large oligonucleotides for target validation in the process of the development of new small analgesic drugs to be generally useful, since the design of antisense agents is much faster and cheaper than the development of chemical substances that act as receptor antagonist. We currently try to improve the strategy by the use of LNA-modified oligonucleotides and siRNA, which have been found to have a higher inhibitory potency than DNA oligonucleotides (Kurreck et al., 2002b; Grünweller et al., 2003; Christoph et al., unpublished results).

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