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Investigation of TRPV1 loss-of-function phenotypes in transgenic shRNA expressing and knockout mice

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The function of the transient receptor potential vanilloid 1 (TRPV1) cation channel was analyzed with RNA interference technologies and compared to TRPV1 knockout mice. Expression of shRNAs targeting TRPV1 in transgenic (tg) mice was proven by RNase protection assays, and TRPV1 downregulation was confirmed by reduced expression of TRPV1 mRNA and lack of receptor agonist binding in spinal cord membranes. Unexpectedly, TRPV3 mRNA expression was upregulated in shRNAtg but downregulated in knockout mice. Capsaicin-induced $[Ca^{2+}]_i$ changes in small diameter DRG neurons were significantly diminished in TRPV1 shRNAtg mice, and administration of capsaicin hardly induced hypothermia or nocifensive behaviour *in vivo*. Likewise, sensitivity towards noxious heat was reduced. Interestingly, spinal nerve injured TRPV1 knockout but not shRNAtg animals developed mechanical allodynia and hypersensitivity. The present study provides further evidence for the relevance of TRPV1 in neuropathic pain and characterizes RNA interference as valuable technique for drug target validation in pain research.

Introduction

TRPV1 is a cation channel that is predominantly expressed by nociceptive sensory neurons. TRPV1 is activated by capsaicin, noxious heat and protons (Caterina et al., 1997; Tominaga et al., 1998). A variety of intracellular pain-related pathways and molecules regulate nociception and signal transmission by TRPV1 (Szallasi et al., 2007; Tominaga, 2007). As TRPV1 appears to be a central molecular integrator of noxious stimuli, it is considered to be an attractive target for new analgesic drugs.

Knockout mice lacking TRPV1 exhibit diminished sensitivity to heat and perturbed micturition (Birder et al., 2002; Caterina et al., 2000; Davis et al., 2000). Interestingly, TRPV1 knockout mice do not reveal a significantly altered phenotype in the partial sciatic nerve ligation model of mononeuropathic pain as compared to wild-type animals (Caterina et al., 2000) and even show increased mechanical hyperalgesia in polyneuropathic pain models (Bolcskei et al., 2005). In contrast, intrathecal administration of the TRPV1 antagonist capsazepine was reported to block A-fiber-evoked responses in dorsal horn neurons of rats after spinal nerve ligation (Kelly and Chapman, 2002), and more selective TRPV1 antagonists attenuated mechanical allodynia and hyperalgesia in rat models of mononeuropathic pain (Christoph et al., 2006; Honore et al., 2005; Kanai et al., 2005; Pomonis et al., 2003). The precise role of TRPV1 in neuropathic pain is therefore still controversial.

We decided to use an RNA interference (RNAi) approach to investigate further the role of TRPV1 in an animal model of neuropathic pain. RNAi is an evolutionary conserved mechanism of posttranscriptional gene silencing mediated by double stranded RNA molecules (Grünweller and Hartmann, 2005; Kim and Rossi, 2007). For applications in mammalian cells, 21 nucleotides long small interfering RNAs (siRNAs) can be employed to specifically silence a particular gene. To achieve long-term inhibition of a target gene, self-complementary short hairpin (shRNAs) can be expressed intracellularly (Shi, 2003). The shRNAs are processed by the RNase Dicer to give siRNA-type molecules. For *in vivo* experiments, transgenic animals have been generated which continuously express the shRNA, thereby permanently knocking down the targeted gene (Seibler et al., 2005).

Several studies have already demonstrated the potential of RNAi to validate new targets in pain research (for reviews, see Ganju and

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Hall, 2004; Röhl and Kurreck, 2006). The pain-related RNAi approaches published to date made use of repeated injections or continuous infusion of siRNA molecules. With this experimental design, however, the knockdown was intended to last only for a limited time of several days. We have also performed some initial functional investigations by transient inhibition of TRPV1 expression by intrathecal injection of antisense oligonucleotides (Christoph et al., 2007) or siRNA molecules (Christoph et al., 2006).

In order to perform a detailed comparison of different gene silencing strategies, we generated transgenic animals that express a TRPV1-directed shRNA and analyzed expression of the transgene, knockdown of TRPV1 and pain-related behaviour. Our study demonstrates that transgenic animals expressing shRNAs can be used for functional investigations in pain research and suggests a role of TRPV1 in neuropathic pain.

Results

Generation of shRNA transgenic mice

In a previous study, we screened for efficient siRNAs targeting the mRNA of TRPV1. One siRNA, named VsiR1, was found to inhibit expression of a TRPV1–GFP fusion construct in the subnanomolar range in co-transfection experiments (Grünweller et al., 2003) and was efficacious upon intrathecal administration in rodent models of mononeuropathic and visceral pain (Christoph et al., 2006). *In vitro* and *in vivo* activities of this particular siRNA have recently been confirmed by an independent group (Kasama et al., 2007).

The siRNA was now converted into a DNA oligonucleotide encoding an shRNA with a loop of 9 nucleotides. In the pRMCE-U6 vector used in the present study, the shRNA was expressed under control of the human U6 promoter. The expression cassette was verified by sequence analysis, and functionality was confirmed in a co-transfection experiment with a plasmid encoding TRPV1–GFP and the pRMCE-U6-shTrpv1 plasmid. Target gene expression was inhibited to almost completion even under conditions with excess of the TRPV1–GFP plasmid (data not shown). In addition, transiently transfected pRMCE-U6-shTrpv1 inhibited capsaicin-induced Ca^{2+} fluorescence in CHO-K1 cells (data not shown).

The RMCE strategy described in Seibler et al. (2005) was used for targeted insertion of an shRNA sequence directed against the Trpv1 mRNA in embryonic stem (ES) cells. The shRNA expression cassette was placed 3′ of the neoR gene on the exchange vector. Upon transfection, recombinase-mediated integration of the exchange vector at the *rosa26* locus was observed in 3 out of 4 analyzed G418 resistant colonies (data not shown). ShRNA transgenic ES cells were injected into tetraploid blastocysts, and ES cell-derived mice were obtained 3 weeks later at a frequency of 5%.

TRPV1 shRNAtg mice were normal regarding breeding and weight gain. Although not specifically tested, animals did not show any overt behavioural differences as compared to the respective controls during the course of the experiment.

RNA analysis

Expression of shRNA was assessed in different organs by RNase protection assays. Total RNA extracts of brain, bladder and liver of several control or TRPV1 shRNAtg animals were incubated with a radioactively labeled probe and subsequently digested with RNase A/T1. The probe is only protected from degradation if the complementary antisense RNA is present in the extracts. As can be seen in Fig. 1, in all RNA extracts derived from brains of different transgenic animals (lanes 6 to 8, 10 to 12, 14 and 15) the antisense strand is present, whereas this is not the case for any of the control animals (lanes 5, 9 and 13). Similar results were obtained with RNA extracts from the non-neuronal tissues bladder and liver (data not shown), clearly proving the ubiquitous expression of the shRNA from the *rosa26* locus. RNase protection assays with defined amounts of siRNA (1 and 10 fmol, respectively) are shown in lanes 2 and 3.

Expression of TRPV1, TRPV3, TRPV4, TRPA1, TRPM8, CGRP and neurofilament was analyzed in DRG neurons of TRPV1 shRNAtg and knockout mice in comparison to their respective control strains by means of real-time PCR. Real-time PCR showed distinct products for all primer pairs, and almost identical efficiencies were obtained for all primer pairs compared to GAPDH (data not shown). There was no difference in expression as compared to control of TRPV4, TRPM8, CGRP and neurofilament (data not shown). A strong downregulation of TRPV1 was demonstrated in TRPV1 shRNAtg (mean expression ratio −3.94, 25.4% remaining expression) and knockout mice (mean expression ratio −4.74, 21.1% remaining expression). Interestingly, TRPV3 expression was regulated differentially. While TRPV3 was upregulated in TRPV1 shRNAtg mice (mean expression ratio 2.18, 218% remaining expression), there was a downregulation in TRPV1 knockout mice (mean expression ratio −1.81, 55% remaining expression).

[^{3}H]-RTX binding sites in spinal cord of TRPV1 shRNAtg and control mice

A binding assay with the TRPV1 agonist resiniferatoxin (RTX) was performed in order to investigate TRPV1 knockdown. Analysis

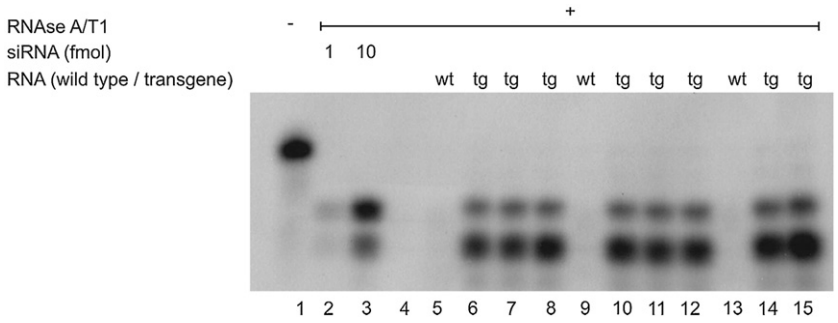

Fig. 1. RNase protection assays with total RNA extracts of mouse brains demonstrate transgene expression. For each hybridization (lanes 5 to 15), 3 μg of total RNA from control (wt) or TRPV1 shRNAtg (tg) animals was incubated for 2 h at 42 °C and treated with RNase A/T1 for 40 min at 37 °C. Lane 1 shows 20% of the full length probe, lanes 2 and 3 show hybridizations with siRNA and lane 4 shows the degradability of the probe.

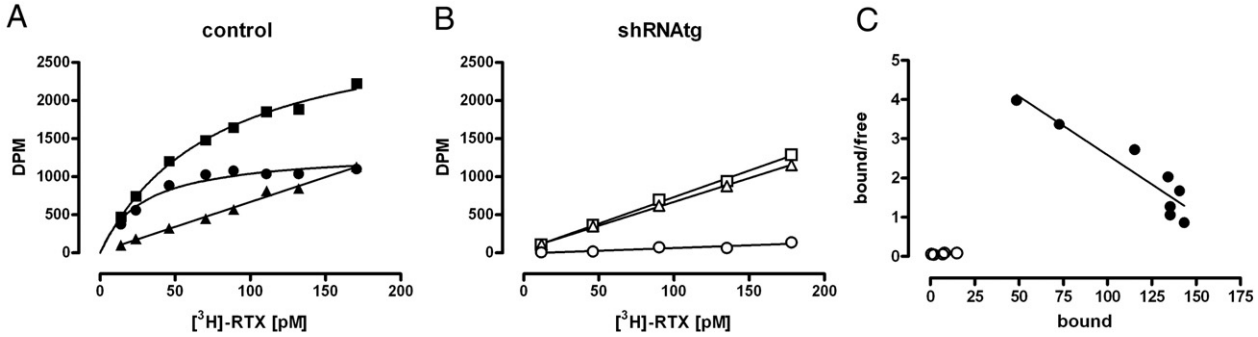

Fig. 2. [^{3}H]-RTX binding of spinal cord homogenates of control (closed symbols, A) and TRPV1 shRNAtg mice (open symbols, B). Maximal (■□), unspecific (▲△) and specific binding (●○) of one representative [^{3}H]-RTX saturation binding experiment is shown. Scatchard plot analysis is shown for the same data sets calculated from bound (fmol/mg) and free (pM) [^{3}H]-RTX (C).

of cervical and thoracal spinal cord membranes of control mice (B6129SF1) revealed a K_d of 39±10 pM and a B_{max} of 114± 22 fmol/mg protein. The specific [^{3}H]-RTX binding was saturable as shown in Fig. 2A. In contrast, neither K_d nor a B_{max} could be determined from the binding data of spinal cord membranes of shRNAtg mice as almost no specific [^{3}H]-RTX binding was detectable (Fig. 2B), which was also shown in comparison to spinal cord membranes of control mice by the respective Scatchard plot (Fig. 2C).

Capsaicin-induced elevation of intracellular calcium in cultured DRG neurons

As can be seen in Fig. 3, perfusion of cultured small diameter DRG neurons of wild-type mice with capsaicin resulted in a significant elevation of the intracellular calcium concentration $[Ca^{2+}]_i$ in 58% (34 out of 59) of the cells, with a median of the increase of the ratio of 0.4 (cell diameter: 20.5±0.6 µm, n=34; IB4 positive: n=29, IB4 negative: n=5). In contrast, only one of 48 (2%) neurons from TRPV1 shRNAtg mice responded to capsaicin resulting in a median

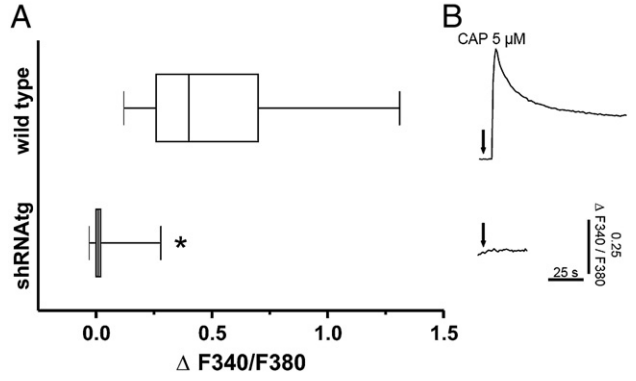

Fig. 3. Capsaicin-induced $[Ca^{2+}]_i$ increase in cultured DRG neurons. Box–Whisker plot of the capsaicin-induced $[Ca^{2+}]_i$ increase in cultured DRG neurons of control and TRPV1 shRNAtg animals (A). Median is indicated by the vertical line, left and right bars represent the lowest and highest value, box indicates the 25–75 percentile. Original traces of capsaicin-induced increase of $[Ca^{2+}]_i$ in cultured DRG neurons of control and TRPV1 shRNAtg mice (B). Small diameter DRG neurons of control and TRPV1 shRNAtg mice were superfused with capsaicin (5 µM, 3 s duration) to activate TRPV1 channels.

of the ratio increase of only 0.01 (cell diameter: 20.0±0.5 µm, n=48; IB4 positive: n=34, IB4 negative: n=14), which was significantly different from the median of capsaicin responsive neurons of control mice (Fig. 3A, P<0.001). Likewise, the median of the ratio increase in capsaicin-unresponsive neurons of control mice of 0.02 (cell diameter: 23.7±0.6 µm, n=25; IB4 positive: n=21, IB4 negative: n=4) was significantly different from the median of capsaicin-responsive neurons of control mice (P<0.001) but was not significantly different from the median of neurons of TRPV1 shRNAtg mice (P=0.42).

To exclude that neurons of TRPV1 shRNAtg and capsaicin-unresponsive neurons of control mice were not responding to capsaicin due to a lack of viability rather than the absence of TRPV1 receptors, a depolarizing stimulus (extracellular solution containing 50 mM K^+) leading to the opening of voltage-dependent Ca^{2+} channels was applied to the cells after capsaicin application. The fluorescence ratio increased in all neurons from TRPV1 shRNAtg mice (median: 0.31; 25th–75th percentile: 0.22–0.51, n=48) and in all capsaicin-unresponsive neurons from control mice (median: 0.57; 25th–75th percentile: 0.33–1.08, n=25) in response to the K^+-induced depolarization demonstrating their viability. Furthermore, capsaicin-sensitive neurons of control mice responded also to high $[K^+]_e$ application with a median of the ratio increase of 0.48 (25th–75th percentile: 0.18–0.57, n=11), which was not significantly different from TRPV1 shRNAtg mice (P=0.70, data not shown).

The biochemical analysis of transgenic mice thus revealed expression of the shRNA against TRPV1 and silencing of the target gene as demonstrated by quantitative PCR and receptor agonist binding assays. Ca^{2+} measurements have further shown a lack of capsaicin-induced increase of the intracellular Ca^{2+} concentration in cultured DRG neurons from the transgenic animals. This finding confirms the absence of functional TRPV1 cation channels in mice expressing the shRNA. After having proven knockdown of the target gene, animals were phenotypically characterized in various pain models and with respect to their response to capsaicin.

Capsaicin-induced effects in vivo

Intraperitoneal administration of the TRPV1 agonist capsaicin is known to have a pronounced effect on body temperature. In wild-type animals, a hypothermic effect was observed that was statistically significant at 7.5, 15 and 30 min after administration of capsaicin (Fig. 4A). In sharp contrast, capsaicin induced only a very short lasting and mild reduction of body temperature in

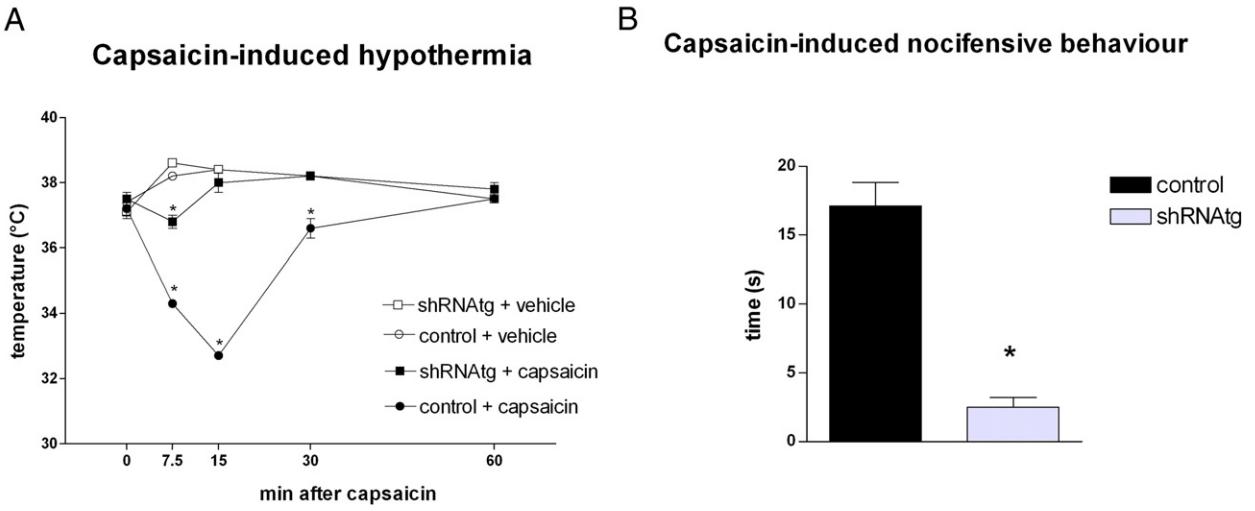

Fig. 4. Analysis of capsaicin-induced effects in control and TRPV1 shRNAtg mice. Body temperature changes (A) upon intraperitoneal administration of 3 mg/kg capsaicin or vehicle in a cross-over design were determined before and 7.5, 15, 30 and 60 min after administration. Data are expressed as mean (±SEM) body temperature in °C. Capsaicin-induced nocifensive behaviour (B) was measured as duration of licking and biting behaviour for 3 min after intraplantar injection of 1 μg capsaicin. Data are expressed as mean (±SEM) time in seconds of licking or biting. *$P<0.05$ capsaicin versus vehicle.

TRPV1 shRNAtg mice. Statistic analysis revealed significance of this effect only at 7.5 min after administration. As expected, vehicle treatment did not have significant impact on body temperature, whereas there was a statistically significant difference when comparing both capsaicin-treated groups ($P<0.001$).

Intraplantar injection of capsaicin induces a strong nocifensive response. This behaviour can be quantified as the time of licking and biting of the hind paw in a certain interval. As can be seen in Fig. 4B, the nocifensive response to the capsaicin stimulus is significantly lower in shRNAtg mice as compared to wild-type animals.

Hot plate test

TRPV1 is known to be the major sensor for noxious heat. Sensitivity of wild-type animals and mice lacking TRPV1 in hot plate assays was therefore compared. Fig. 5 shows that latency times of the transgenic mice towards relatively weak (48 °C) and relatively strong (58 °C) thermal stimuli was significantly increased. This finding clearly shows a reduction of sensitivity to thermal stimulation as a consequence of TRPV1 knockdown.

Phenotypic analyses performed so far demonstrate a diminished response to stimuli with the receptor agonist capsaicin as well as reduced thermal sensitivity in animals inhibiting TRPV1 expression by means of RNAi. These behavioural findings are consistent with the known function of TRPV1. They are furthermore comparable to observations made with TRPV1 knockout mice (Caterina et al., 2000). As outlined in the introduction, inconsistencies exist in the literature with respect to the relevance of TRPV1 in neuropathic pain. We next aimed at clarifying this important point.

Spinal nerve ligation

Nerve injury models have been developed to investigate neuropathic pain in rodents. We wanted to analyze the influence of TRPV1 knockdown on the development of mechanical hypersensitivity and mechanical allodynia. In control mice, spinal nerve

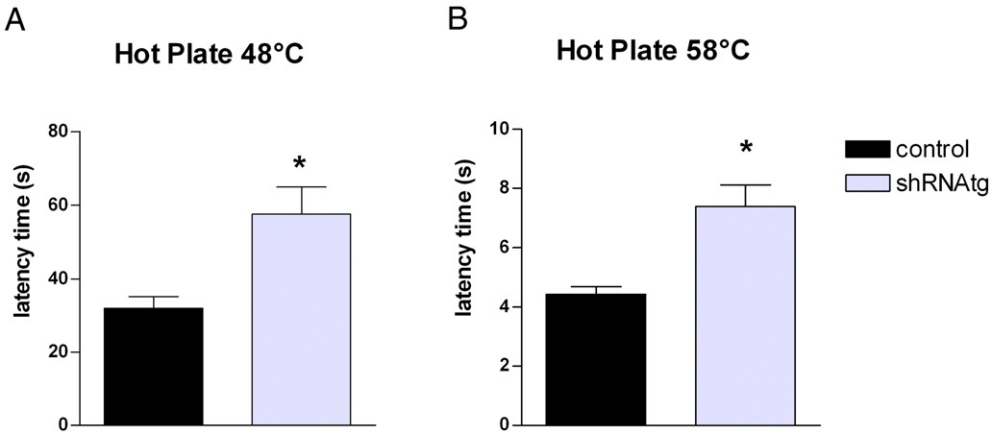

Fig. 5. Analysis of heat-induced nociception in control and TRPV1 shRNAtg mice. Latency times were determined in a 48 °C (A) and 58 °C hot plate test (B). Data are expressed as mean (±SEM) latency time in seconds. *$P<0.05$ versus controls.

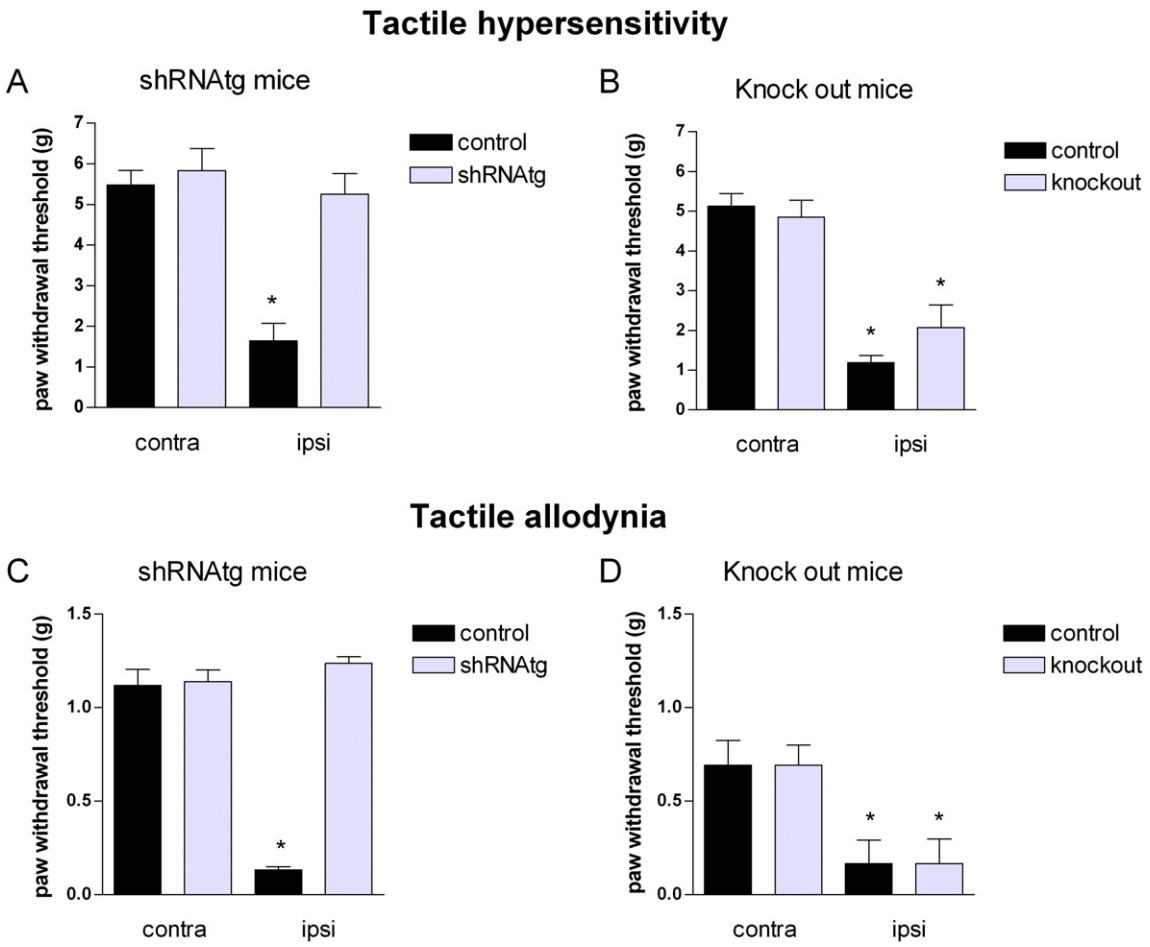

Fig. 6. Analysis of mechanical hypersensitivity and allodynia after spinal nerve ligation in control, TRPV1 shRNAtg and TRPV1 knockout mice. Animals were tested on the ipsi- and contralateral hind paw during week 1 after operation with electronic (A, B) and during week 2 after operation with mechanical von Frey filaments (C, D). Data are expressed as mean (±SEM) paw withdrawal threshold in grams. *$P<0.05$ versus contralateral paw.

ligation resulted in the development of mechanical hypersensitivity as demonstrated by the occurrence of hypersensitivity of the ligated paw (as compared to the non-ligated contralateral paw) upon stimulation with an electronic von Frey filament 1 week after operation (Fig. 6A, black bars). Similar observations were made 3 weeks after operation (data not shown). Likewise, mechanical allodynia was demonstrated 2 weeks after operation by means of mechanical von Frey filaments (Fig. 6C, black bars). In contrast, mice expressing the shRNA against TRPV1 showed neither mechanical hypersensitivity nor mechanical allodynia when tested at the same time points after spinal nerve ligation (Figs. 6A, C, grey bars).

While these results that suggest functional relevance of TRPV1 for the development of mechanical hypersensitivity and allodynia are supported by published data obtained with low molecular receptor antagonists, they are in conflict to findings reported for TRPV1 knockout animals (Caterina et al., 2000). We therefore tested the knockout animals in our experimental setting. One week after operation, control mice as well as TRPV1 knockout mice showed clear signs of mechanical hypersensitivity on the ligated paw (as compared to the non-ligated contralateral paw) upon stimulation with an electronic von Frey filament (Fig. 6B). Again, mechanical allodynia was measured 2 weeks after operation by means of mechanical von Frey filaments. Both control and TRPV1 knockout animals developed mechanical allodynia (Fig. 6D), which confirms published data (Caterina et al., 2000).

Discussion

The present study successfully demonstrates that RNAi is a useful approach to validate potential novel targets for pain therapy. Previous studies have shown that the knockdown of the expression of several pain-related genes, such as the delta opioid receptor (Luo et al., 2005), the ionotropic nucleotide receptor P2X3 (Dorn et al., 2004), the NMDA receptor NR2B subunit (Tan et al., 2005) or TRPV1 (Christoph et al., 2006), by acute or sub-acute local administration of siRNAs resulted in analgesic effects in various animal models of nociception. The siRNA characterized in our previous studies (Christoph et al., 2006; Grünweller et al., 2003) has recently been used by an independent group to demonstrate that knockdown of TRPV1 prevents the development of inflammatory thermal hyperalgesia (Kasama et al., 2007).

As the pre-synthesized siRNAs of the abovementioned studies cover a whole range of doses and affinities, it is not easy to predict a suitable level for intracellular shRNA expression. Based on the strong potency *in vitro* and *in vivo* of our previously described siRNAs against TRPV1 (Christoph et al., 2006; Grünweller et al., 2003), we decided to use a system with moderate shRNAtg expressions levels. A

single copy of the expression cassette was inserted by RMCE into the *rosa26* locus, which is known to be accessible in a variety of tissues. TRPV1 shRNAtg expression was detectable in both neuronal and non-neuronal tissues. Since no obvious behavioural side effects were observed, it is reasonable to assume that *rosa26* is an appropriate locus for the insertion of expression cassettes without the risk of excessive, deleterious expression levels, which may occur after viral delivery of expression cassettes (Grimm et al., 2006).

Previous studies have elegantly demonstrated that TRPV1 knockout mice are viable and fertile but show impaired nociception and bladder function (Birder et al., 2002; Caterina et al., 2000; Davis et al., 2000). In these studies, lack of TRPV1 protein was confirmed by immunocytochemistry and functional approaches. In our current study, downregulation of TRPV1 protein was demonstrated in spinal cord homogenate by determination of [^{3}H]-RTX binding by saturation analysis. RTX, as an ultra-potent capsaicin analogue, has frequently been used for TRPV1 binding (Szallasi and Blumberg, 1993). The fact that TRPV1 shRNAtg mice showed almost no specific binding of [^{3}H]-RTX strongly suggest a lack of TRPV1. Likewise, real-time PCR expression analyses showed comparable downregulation of TRPV1 in both TRPV1 shRNAtg and knockout mice. Remaining TRPV1 mRNA expression might occur from the naive TRPV1 gene in shRNAtg mice and from the targeted TRPV1 gene in the knockout mice carrying a neomycin expression cassette in the transmembrane region (Caterina et al., 2000).

TRPV1 receptors are unspecific cation channels with high permeability for Ca^{2+} ions. To investigate the downregulation of functional TRPV1 receptors in shRNAtg mice, we used cultured small diameter DRG neurons of wild-type control and shRNAtg mice for imaging experiments of the intracellular Ca^{2+} concentration. While only 2% of IB4-positive DRG neurons were reported to be TRPV1 immuno-positive in B6C3HF1 mice (Zwick et al., 2002), we found that 58% (29 out of 50 cells) of IB4-positive DRG neurons expressed functional TRPV1 receptors in B6129SF1 wild-type mice, resembling published ratios from other rodents (Guo et al., 1999; Liu et al., 2004). A significantly diminished increase of the intracellular Ca^{2+} level after application of capsaicin demonstrated functional loss of TRPV1 activity in shRNAtg DRG neurons for both IB4-positive and -negative populations. Therefore, it is reasonable to assume that small diameter DRG neurons of TRPV1 shRNAtg mice, which are viable as indicated by their response to a high K^+ stimulus, include cells which would express TRPV1 in a normal state.

Close similarity of TRPV1 shRNAtg with TRPV1 knockout mice was not only seen *in vitro* but also in standard assays of capsaicin and heat-evoked responses *in vivo*. Systemic administration of capsaicin generates transient hypothermia, which is absent in TRPV1 knockout mice (Caterina et al., 2000). Interestingly, TRPV1 shRNAtg mice showed a similar phenotype, but a very transient and mild hypothermia after capsaicin administration was still observed. The presence of this residual capsaicin-induced hypothermia might have several reasons. First, capsaicin was given at a higher dose and different route of administration (3 mg/kg i.p.), as compared to the report using knockout mice (1 mg/kg s.c.). Second, transgene expression might be variable since two out of ten shRNAtg mice showed differences in the degree of capsaicin-induced hypothermia, leading to a significant decrease in body temperature 7.5 min after administration of capsaicin. Finally, residual TRPV1 protein might be translated since real-time PCR data suggest the existence of low levels of remaining TRPV1 mRNA, which might escape RNA interference.

Consistent with the findings reported in TRPV1 knockout mice (Caterina et al., 2000), shRNAtg mice hardly showed capsaicin-induced licking and biting (Fig. 4B), demonstrating again that TRPV1 was functionally affected *in vivo*. When analyzed in a hot plate test, response latency times of TRPV1 knockout mice were significantly increased at temperatures of >50 °C, indicating that sensitivity to noxious thermal stimuli was decreased (Caterina et al., 2000). Again, a similar phenotype was observed in TRPV1 shRNAtg mice, as we found that latency times were significantly increased at 48 °C, a stimulus intensity well above the known thermal threshold of TRPV1 of >42 °C (Caterina et al., 1997).

The role of TRPV1 in neuropathic pain has been discussed controversially (Caterina et al., 2000; Maione et al., 2006; Pomonis et al., 2003). Being a heat-stimulated receptor, TRPV1 is expected to be involved in heat hyperalgesia resulting from nerve injury. This is presumably achieved via C-fibers and the fact that TRPV1 antagonists attenuate thermal nociceptive responses support this hypothesis (Jhaveri et al., 2005). On the other hand, heat hyperalgesia could still be observed after partial nerve injury in TRPV1 knockout mice (Caterina et al., 2000). Likewise, the situation is unclear with respect to mechanical hypersensitivity. Neuropathic pain-induced mechanical allodynia was shown to be dependent on A-fibers rather than C-fibers since capsaicin-treated C-fiber-depleted rats develop mechanical allodynia after spinal nerve ligation (Ossipov et al., 1999). Thus, it can be argued that TRPV1, which usually is expressed predominantly on C-fibers, would not be expected to be involved in the development of mechanical allodynia. Indeed, the finding that TRPV1 knockout mice do not show a deficit in the development of mechanical allodynia after nerve ligation as reported by Caterina et al. (2000) and shown in Fig. 6 of the present study seems to support this hypothesis. Furthermore, administration of the TRPV1 antagonist iodoresiniferatoxin had no effect on mechanically evoked responses of wide dynamic range neurons in rats after spinal nerve ligation (Jhaveri et al., 2005). Moreover, TRPV1 expression is known to be downregulated in peripherally injured nerves (Michael and Priestley, 1999). However, it should be noted that TRPV1 expression has not only been reported to be downregulated in neuropathic pain states. It was, for example, shown that TRPV1 is upregulated in undamaged DRG neurons after peripheral nerve injury (Hudson et al., 2001), and even TRPV1 *de novo* expression in otherwise TRPV1-negative A-fibers appears to take place under these conditions (Rashid et al., 2003). Therefore, it is still conceivable that TRPV1 plays a role in mechanical allodynia. Indeed, it has been reported that selective TRPV1 antagonists are able to attenuate mechanical allodynia in various neuropathic pain models (Drizin et al., 2006; Honore et al., 2005; Kanai et al., 2005; Pomonis et al., 2003). In line with these pharmacological studies, our data obtained with transgenic mice expressing an shRNA against TRPV1 support the hypothesis that TRPV1 contributes to mechanical allodynia and hyperalgesia.

Differences in methodology can be ruled out as explanation for the inconsistency between RNAi-mediated knockdown and conventional knockout approaches since TRPV1 knockout mice develop tactile allodynia and hypersensitivity in our experimental setting as previously reported (Caterina et al., 2000). As suggested by residual capsaicin-induced hypothermia, some functional TRPV1 activity might be retained in TRPV1 shRNAtg mice. The complete absence of TRPV1 in knockout mice might induce so far undetected compensatory changes responsible for the induction and maintenance of mechanical allodynia and hypersensitivity. On the other hand, residual TRPV1 activity as hypothesized for the TRPV1 shRNAtg mice might prevent these changes and thus resemble a more physiological

situation. Interestingly, similar observations were made in a comparative study with knockout animals and mice treated with siRNAs targeting the peroxisome proliferator-activated receptor alpha (Ppara) (De Souza et al., 2006). RNAi-mediated silencing of Ppara was found to result in a transcript profile and phenotype that were highly comparable to that observed in Ppara$^{-/-}$ mice. Marked differences, however, were observed for one parameter since siRNA-treated animals developed hypoglycemia and hypertriglyceridemia in the absence of fasting, an observation that was not made with knockout animals. It should be noted that De Souza et al. (2006) employed hydrodynamic tail vein injection to deliver siRNAs. This method is characterized by the rapid injection of a large volume of the siRNA solution and is known to be a harsh treatment that can cause severe side effects. For our present study, we used two types of transgenic animals, i.e., shRNA expressing and knockout mice. Differences caused by the methodology for the generation of the loss-of-function phenotypes are therefore less likely.

In an attempt to find a molecular basis for the phenotypic differences between TRPV1 knockout and shRNAtg animals in the present study, we analyzed expression of other pain-related TRP channels and markers of sensory neurons. We did not observe significant differences for any of the analyzed mRNAs demonstrating the specificity of the approach. Only TRPV3 is upregulated in TRPV1 shRNAtg mice, while it is downregulated in knockout mice. Although the mouse gene encoding TRPV3 is only 9 kb away from the gene encoding TRPV1 (Peier et al., 2002), a direct link between expression of both genes seems to be unlikely. It should be realized, however, that TRPV1 knockout mice still contain the neo-selection marker (Caterina et al., 2000), which might have induced TRPV3 gene silencing (Olson et al., 1996). Off-target effects have been reported for RNAi approaches as well (Jackson and Linsley, 2004), but these unspecific effects appear to result in unintended downregulation of mRNAs with partial homology to the siRNA (Birmingham et al., 2006). Since TRPV3 is, in contrast, upregulated in the transgenic shRNA expressing animals, this is unlikely to be an unspecific effect caused by the siRNA. There is no sufficient homology between the TRPV1 shRNA used in this study and the TRPV3 to justify off-target regulation, and comparable transgenic mouse lines targeting different genes give no indication on off-target activation of target-unrelated mRNAs (Seibler et al., 2007, 2005). On the other hand, it is possible that developmental compensation could be responsible for the upregulation of TRPV3 in shRNAtg mice (Arnold and Braun, 1996; Maddison and Clarke, 2005). This finding would lead to the suggestion that knockdown as well as knockout mice should be preferably generated in a conditional manner (Seibler et al., 2007; Seibler et al., 2003). Moreover, it can be argued that incomplete knockdown by shRNAs is more likely to reflect the situation that occurs after systemic administration of low molecular antagonistic drugs, which eventually also leads only to an imperfect blockade of the molecular target.

It was reported previously that TRPV3 knockout mice show deficits in response to heat stimuli, suggesting that TRPV3 is involved in acute thermal nociception (Moqrich et al., 2005). Since both TRPV1 shRNAtg and knockout mice show increased latency times for acute heat nociception, we do not consider the slightly different regulation of TRPV3 in both models to be of major importance. However, differences were noted in response to mechanical rather than heat stimulation under neuropathic pain conditions, and therefore further studies are needed to analyze the potential relationship between TRPV3 and mechanical allodynia and hypersensitivity. Recent evidence demonstrating increased expression of TRPV3 in human injured peripheral nerves is in line with these data and suggests a role for TRPV3 in neuropathic pain states (Facer et al., 2007).

It can be concluded that transgenic mice expressing shRNA offer a powerful tool for target validation in pain research. Classically, the generation of congenic knockout mice is a time-consuming process, whereas the RMCE technology to transfer shRNA expression cassettes employed in the present study allows the generation of knockdown animals within only a few months. Transgenic mice expressing an shRNA against TRPV1 resemble knockout mice in expression, capsaicin-evoked responses and nociceptive thresholds to heat. Of particular importance, however, is the finding that TRPV1 shRNAtg mice hardly show mechanical allodynia and hyperalgesia under neuropathic pain conditions. This supports the hypothesis that TRPV1 is involved in neuropathic pain and that TRPV1 antagonists may offer a novel therapeutic approach to treat these debilitating symptoms.

Experimental methods

Construction and testing of the shRNA expression vector

For the generation of transgenic animals, an exchange vector, named pRMCE-U6-shTrpv1, was constructed that encodes an shRNA under control of the U6 promotor: The vector contains the F3 site and the FRT site in the same configuration as in the rosa26 targeting vector described by Seibler et al. (2005). The vector was generated using standard cloning procedures and has the following order in 5′ to 3′ direction: synthetic polyA signal, F3 site and a neomycin resistance gene lacking the start ATG. The U6-promoter fragment was amplified from human genomic DNA with attached *Bbs*I/*Avr*II sites and cloned into the basic exchange vector (pRMCE-U6). The Trpv1-specific shRNA sequence (gcgcatcttctacttcaac (sense) TTAGCACTG (loop) gttgaagtagaagatgcgc (antisense)) together with five thymidines was generated by oligonucleotide annealing and inserted into pRMCE-U6 upon *Bbs*I/*Avr*II restriction. Insertion at the *Bbs*I site in pRMCE-U6 allowed transcription of the shRNA starting at the first base of the hairpin sequence.

Cell culture

F1-ES cells were cultured as previously described (Eggan et al., 2001; Seibler et al., 2005). Transfection of recombinase-mediated cassette exchange (RMCE) cells with the pRMCE-U6-shTrpv1 for RMCE has been performed as outlined in Seibler et al. (2005).

Animals

Recombinant shRNA ES cell clones were injected into tetraploid blastocysts (Eggan et al., 2001), and 8-week-old ES mice were derived within 3 months. B6D2F1 mice for the generation of tetraploid blastocysts were obtained from Harlan (Horst, The Netherlands). Mice were kept in the animal facility at Artemis Pharmaceuticals (Cologne, Germany) in microisolator cages (Tecniplast Sealsave). TRPV1 knockout mice (B6.129S4-Trpv1^{tm1Jul}/J, Jax mice, USA) were used on congenic background and compared to C57BL/6J mice (Charles River, Sulzfeld, Germany).

Studies were conducted with male mice (20–35 g). Animals were housed at Grünenthal (Aachen, Germany) under standard conditions (room temperature 20–24 °C, alternating 12-h light/dark cycle, relative air humidity 45–70%, 15 air changes per hour, air movement <0.2 m/s) with food and water available ad libitum, except for the time of the experiment. Thirty-four shRNAtg and 34 control animals (B6129SF1) were used. Behavioural experiments were performed in the following order with 1 week washout time: group 1: hot plate 48 °C, hot plate 58 °C, spinal

nerve ligation; group 2: capsaicin-induced hyperthermia, capsaicin-induced nocifensive behaviour; group 3: preparation of dorsal root ganglia (DRG), brain, spinal cord and selected other organs. There were at least 5 days between delivery of the animals and the first test day. Seven TRPV1 knockout and 11 control mice (C57BL/6) were used in the spinal nerve ligation model, 5 TRPV1 knockout and 5 control mice (C57BL/6) were used for DRG preparation and subsequent real-time PCR. All animal studies were performed in accordance with the recommendations and policies of the International Association for the Study of Pain (Zimmermann, 1983) and the German Animal Welfare Act. All study protocols were approved by the local government committee for animal research, which is also an ethics committee.

Assessment of RNA expression

Total RNA from different organs was extracted by Miltenyi Biotec (Cologne, Germany). Tissues were homogenized in liquid nitrogen prior to the extraction of RNA with 2 ml TriReagent (Sigma, St. Louis, MO) per 100 mg of tissue, following the manufacturer's instructions. RNA was quantified by photometrical measurement (Nanodrop) and the integrity proven by the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Expression of the shRNA was confirmed with an RNase protection assay (Overhoff et al., 2004). A sense RNA probe was generated by in vitro transcription employing the mirVANATM miRNA Probe Construction Kit (Ambion, St. Austin, TX). An oligonucleotide with the sequence 5′-gttgaagtagaagatgcgcttcctgtctc-3′ (TIB Molbiol, Berlin, Germany) was used to generate the template and [^{32}P]-α-CTP with the specific activity of 800 Ci/mmol (GE Healthcare, Munich, Germany) was added for the labeling reaction. After gel purification (15% PAGE/7 M urea/1× TBE) of the probe, 5×104 cpm were used for each hybridization reaction performed with the mirVANATM miRNA Detection Kit (Ambion, St. Austin, TX). The assays were separated (15% PAGE/7 M urea/1× TBE) and analyzed by X-ray film exposure. Expression of TRPV1, TRPV3, TRPV4, TRPA1, TRPM8, CGRP and neurofilament was analyzed in comparison to a house keeping gene (GAPDH) in DRG of TRPV1 shRNAtg and knockout mice and their respective control strains by means of real-time PCR (Miltenyi Biotec, Cologne, Germany). Pooled DRGs from five mice of each strain were used for RNA isolation, and 1 μg of each RNA was reverse transcribed into cDNA. Primer pairs for the seven target genes and GAPDH were designed and experimental conditions for real-time PCR (SDS7000, ABI, Applied Biosystems) were established. Primer pairs were as follows (5′–3′):

GAPDH	forward	ACCTGCCAAGTATGATGACATCA
	reverse	TGCTGTTGAAGTCGCAGGAGA- CAA
TRPV1	forward	ATGCTGGGTCATTTCTCCCC
	reverse	CAGCGTACCCCCACTTTCTCT
TRPV3	forward	CATCACCCTGACCCTTGTCTC
	reverse	CCCTAGGAGCTGAAGCCAACT
TRPV4	forward	AGGGCTACGCTCCCAAGTG
	reverse	CACCGGACAAATGCCTAAATG
TRPA1	forward	ATCCAAATAGACCCAGGCACG
	reverse	CAAGCATGTGTCAATGTTTGGTA- CT
TRPM8	forward	GATCGCCAAGAACTCCTACAATG
	reverse	CCCTGCTGCTTCTGTCCTCT
CRCP	forward	GCAGGTCCAGAGGATGAACAG
	reverse	CTTCACGAACAACTTGGCCA
neurofilament	forward	AAAGAAACTCAGGACGGTCCC
	reverse	CAGGCCCACCATCTAAGCAG

Each primer had to fulfill the following conditions, melting temperature between 58 °C and 60 °C, length between 17 and 25 base pairs, GC content between 40% and 60%, length of the amplified product between 100 bp and 130 bp and cross hybridization being excluded. Melting curve analysis was performed for all amplified products. Six nanograms of cDNA and 200 nM of each primer were used per reaction. Having proven equal efficiencies for target and house keeping genes, Ct-values (cycle over threshold) were determined in triplicates in two independent experiments. Expression profiles were generated by the delta delta Ct method (Livak and Schmittgen, 2001). In a first step, the difference between the Ct-value of target gene and house keeping gene were measured in each "treated" sample (i.e., TRPV1 shRNAtg and TRPV1 knockout; delta target). In every control sample (i.e., B6129SF1 and C67BL/6), the difference between the Ct-value of target gene and house keeping gene was calculated the same way (delta control). Subsequently, the differences between "delta target" and "delta control" were calculated and the relative expression was determined (1 delta delta Ct corresponds to 2-fold regulation). Mean expression ratios from two independent experiments are given.

[^{3}H]-RTX binding studies

The [^{3}H]-resiniferatoxin (RTX) binding at mouse spinal cord membranes was essentially done according to Szallasi and Blumberg (1993). Briefly, mice were anesthetized by blow on the back of the neck and killed by decapitation. Spinal cord was dissected from the cervical and the thoracic spinal cord segments and homogenized by means of a tissue homogenizer in ice-cold assay buffer, 10 mM HEPES, 5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 12 mM D-glucose and 137 mM sucrose (pH 7.4) for 2 min at 6000 rpm at 4 °C. The supernatant of a preceding centrifugation for 10 min with 1000×*g* was again centrifuged for 40 min at 48000×*g* and 4 °C. After resuspension of the resulting pellet with a tissue homogenizer in assay buffer for 1 min at 6000 rpm and 4 °C, the membrane fraction was aliquoted and kept frozen at −80 °C until assay. For the K_d determinations, the spinal cord membranes were used at a concentration of about 50–65 μg protein/ml. Spinal cord membranes of either B6129SF1 control mice (n=4) or TRPV1 shRNAtg mice (n=4) were incubated for 1 h at 37 °C in polyethylene test tubes with a total volume of 3 ml assay buffer supplemented with 25 μg/ml bovine serum albumin. [^{3}H]-RTX concentration range for the saturation analysis was 12.5–200 pM, addition of 1 μM unlabeled RTX served as unspecific binding control. Immediately after incubation, the test tubes were placed on ice for further 20 min before addition of 100 μg/ml of α_1-acid glycoprotein for reduction of unspecific binding of [^{3}H]-RTX during the filtration process. After further 10 min incubation at 4 °C, the incubates were filtrated through pre-soaked Brandel GF/B glass fibre filter mats (30 min with ice-cold assay buffer) by means of a Brandel cell harvester and were washed thrice with 5 ml ice-cold assay buffer. All labware used for dilution and incubation of test reagents was siliconized prior to use.

Culture of DRG cells

Adult mice were killed by CO_2 and decapitation. Spinal cords of three to four mice were removed quickly and collected in cold Hanks' balanced salt solution (HBSS)+1% AA-solution. Lumbar DRGs from segments L4–L6 were removed and chilled in cold HBSS+1% AA-solution. Ganglia were freed from connecting tissue, transferred to collagenase solution and incubated for 45 min at 37 °C in an incubator. Trypsin was added (2.5%) for further 15 min of incubation. After the incubation, enzyme solution was removed and culture medium (HBSS containing 10% FCS, 2 mM L-glutamine, 1% AA-solution, NGF 25 ng/ml) was added to deactivate enzymes. Cell suspension was triturated several times and passed through a cell strainer (mesh size 70 μm) to remove undigested tissue fragments. After centrifugation for 10 min at 1200 rpm, the pellet was resuspended in the culture medium. DRG cells were plated on poly-D-lysine-coated glass cover slips with a density of 3×10^5 cells/ml. Cultures were maintained for 1 to 3 days in a humidified atmosphere (37 °C, 5% CO_2) before experimentation.

Intracellular calcium measurements

DRG cells were incubated at 37 °C in the dark with staining solution consisting of extracellular solution (ECS) containing 1 μM of the cell permeant acetoxymethyl ester of the fluorescent Ca^{2+} indicator Fura-2 and FITC-labeled *Bandeiraea simplicifolia* isolectin IB4 (6 μg/ml). The ECS

consisted of the following (in mM): 140 NaCl, 4.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES (pH adjusted to 7.4 with NaOH). The staining solution was removed after 50 to 60 min, and glass cover slips were washed twice with ECS. Cells were allowed to rest for 15–30 min at room temperature protected from light. Thereafter, Ca^{2+} imaging experiments were performed at room temperature in ECS using an upright microscope (E600FN, Nikon, Cologne, Germany) equipped for epifluorescence and a Peltier-cooled charge-coupled device camera (IMAGO; Till Photonics Inc., Martinsried, Germany). Intracellular Fura-2 was alternately excited at 340 nm and at 380 nm, and the emitted light was measured at a wavelength of 510 nm. The TILL Vision software was used for data acquisition, system control and later for off-line data analysis. The experimental protocol routinely used started with the application of ECS for 10 s via a pressure-operated, computer-controlled rapid drug application device (ALA DAD-12; HEKA, Lambrecht, Germany), followed by the application of capsaicin (5 μM) for 3 s and another application of ECS for 10 s to achieve a rapid washout of capsaicin. The very slow decay of effect and the often incomplete return to baseline, even after a washout period of 5 min, restricted the use of capsaicin to one application. After a washout period of 5 min, ECS with an elevated K^+ concentration (50 mM) was applied to activate high-voltage-activated calcium channels. In this solution, the sodium concentration was reduced equimolarly to ensure constant osmolarity. Because DRG neurons generally express high-voltage-activated calcium channels (Petersen and LaMotte, 1991; Scroggs and Fox, 1992), only neurons of shRNAtg mice positively tested for excitability and viability by depolarization with a high K^+ solution were included in the evaluation of data. Data were given as the fluorescence ratio of wavelength of 340 nm and 380 nm (F340/F380). Baseline values were subtracted from peak values of F340/F380 and therefore given as ΔF340/F380. Results were expressed as median values of *n* experiments if not indicated otherwise.

Behavioural studies

Animals were assigned randomly to treatment groups. Each experimental model was performed with 10 TRPV1 shRNAtg and 10 control mice, unless otherwise stated. Different groups within one experimental model were tested in a randomized fashion. Although the operators performing the behavioural tests were not formally "blinded" with respect to the treatment, they were not aware of the study hypothesis.

Capsaicin-induced hypothermia

Intraperitoneal injection of capsaicin 3 mg/kg or vehicle (5% DMSO, 5% Cremophor EL, in 5% glucose) was used to analyze TRPV1 agonist-induced hypothermia. All animals tested received capsaicin and vehicle in a cross-over design in randomized order with 1 week washout period. The rectal temperature was measured twice before (baseline) and 7.5, 15, 30 and 60 min after capsaicin with a thermocouple probe (connected to a digital thermometer; Thermalert TH-5, physitemp, Clifton NJ).

Capsaicin-induced nocifensive behaviour

Capsaicin-induced nocifensive behaviour (adapted from Sakurada et al., 1992) was carried out in an open glass box with a mirror placed behind to allow an unobstructed view of the animals. Each animal was injected with 1 μg capsaicin/20 μl vehicle (2% ethanol; 2% Tween 80; in 0.9% NaCl) subcutaneously into the dorsal surface of the right hind paw. After placing the mice back into the chamber, they were observed during the time interval of 0 to 3 min post-capsaicin, and the time spent licking and biting the injected paw was monitored.

Hot plate test

The hot plate test was adapted from Eddy and Leimbach (1953). The device consisted of an electrically heated surface and an open plexiglas tube (17 cm high × 22 cm diameter) to confine the animals to the heated surface. The temperature was kept at either 48.0 ± 0.5 °C or 58.0 ± 0.5 °C (48 °C and 58 °C hot plate test, respectively) in order to analyze supraspinally and spinally evoked nocifensive reactions, respectively. Mice were placed on the hot plate and the time until either licking of the hind paw or jumping occurred was recorded with a stopwatch. On the 48 °C hot plate, animals were tested twice; on the 58 °C hot plate, animals were tested once before baselines were taken. Testing interval was 30 min, respectively. The cut-off time were 120 s (48 °C) and 15 s (58 °C). There was a 1-week washout period between both tests.

Spinal nerve ligation

The spinal nerve ligation model of neuropathic pain was adapted from Kim and Chung (1992) and performed in the animals which were previously exposed to the hot plate (washout period of 1 week). Under pentobarbital anesthesia (Narcoren®, 60 mg/kg i.p.), the left L5, L6 spinal nerves were exposed by removing a small piece of the paravertebral muscle and a part of the left spinous process of the L5 lumbar vertebra. The L5 and L6 spinal nerves were then carefully isolated and tightly ligated with silk (NC-silk black, USP 5/0, metric 1, Braun Melsungen, Germany). Muscle and adjacent fascia were closed with sutures, and the skin was closed with metal clips. After surgery, animals were allowed to recover for 1 week. For the assessment of mechanical allodynia, mice were placed on a metal mesh covered with a plastic dome and were allowed to habituate until exploratory behaviour ceased. Thresholds were measured for mechanical allodynia with calibrated von Frey filaments (0.005, 0.023, 0.028, 0.068, 0.166, 0.407, 0.692, 1.202 and 1.479 g) 2 weeks after operation by the "up and down" method according to Chaplan et al. (1994), 1 week for the mechanical hypersensitivity with an electronic von Frey anesthesiometer (Somedic AB, Sweden) and 3 weeks in the case of TRPV1 shRNAtg mice and their controls after operation on both the ipsi- and the contralateral paw, respectively. Data were expressed as mean paw withdrawal threshold in grams. Group size was $n=8$ for TRPV1 shRNAtg and $n=9$ for control animals. Analysis of TRPV1 knockout animals was performed with $n=7$ for TRPV1 knockout and $n=11$ for control animals.

Data analysis

K_d and B_{max} were calculated by means of the program GraphPadPrism™ for Windows™, version 4.03, from GraphPad Software Inc., San Diego, and are given as mean ± SEM. *In vitro* data from cultured DRG neurons were analyzed by Mann–Whitney test. *In vivo* data were analyzed by repeated measures ANOVA and post hoc Bonferroni test or by ANOVA and post hoc Dunnett test. Results were considered statistically significant if $P<0.05$.

Drugs and chemicals

Phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), nutrient mixture Hams' F12, nerve growth factor (NGF), laminin and Fura-2-AM (acetoxymethyl ester) were obtained from Invitrogen-Molecular Probes (Karlsruhe, Germany). Collagenase type 2, trypsin, antibiotic–antimycotic solution (AA-solution) and fetal calf serum (FCS) were obtained from PAA Laboratories (Coelbe, Germany). L-Glutamine, poly-D-lysine (PDL) and FITC-labeled *B. simplicifolia* isolectin IB4 were obtained from Sigma-Aldrich (Deisenhofen, Germany). Capsaicin was obtained from Tocris-Biorend Chemikalien (Cologne, Germany). Sterile disposable plasticware for cell culture was obtained from Greiner (Frickenhausen, Germany).

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