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## **Vanilloid receptor-1 (TRPV1) expression and function in the vasculature of the rat**

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## Summary

Transient receptor potential (TRP) cation channels are emerging in vascular biology. In particular, expression of the capsaicin receptor (TRPV1) was reported in vascular smooth muscle cells. This arteriolar TRPV1 function and expression was characterized here in the rat.

TRPV1 mRNA expressed in various vascular beds. Six commercially available antibodies were tested for TRPV1 specificity. Two of them was specific (immunostaining was abolished by blocking peptides) for neuronal TRPV1 and one of these antibodies recognized vascular TRPV1. TRPV1 was expressed in blood vessels in the skeletal muscle, mesenteric and skin tissues, besides to the aorta and carotid arteries. TRPV1 expression was found to be regulated at the level of individual blood vessels (some vessels expressed, while others did not express TRPV1 in the same tissue sections). Capsaicin (a TRPV1 agonist) evoked constrictions in skeletal muscle arteries and in the carotid artery, while it did not have effects in femoral and mesenteric arteries and in the aorta.

TRPV1 expression in blood vessels was detected in most large arteries, but there are striking differences at level of the small arteries. TRPV1 activity was suppressed in some isolated arteries. This tightly regulated expression and function suggests a physiological role for the vascular TRPV1.

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## Introduction

Capsaicin is the most irritative component in hot chilli peppers (Szallasi and Blumberg 1999). The receptor which mediates the hot painful feeling upon capsaicin exposure (transient receptor potential channel vanilloid 1, TRPV1) was cloned and identified in sensory neurons (Tominaga et al. 1998). The physiological effects of capsaicin identified TRPV1 as a promising therapeutic target to modulate pain perception and an extensive pharmaceutical effort was made to develop TRPV1 antagonists to relieve pain (Szallasi and Blumberg 1999). There are hundreds of patents and thousands of molecules developed to modulate TRPV1. However, this effort did not result in a breakthrough in pain treatment, which is related to the physiological effects of the developed TRPV1 antagonists. It appears that TRPV1 is involved in body temperature maintenance besides other functions, giving rise to on-target side effects (Gavva 2008; Holzer 2008; Szallasi and Sheta 2012).

TRPV1 expression has been identified in various tissues in addition to sensory neurons. In particular, TRPV1 was found in the central nervous system (Toth et al. 2005) and in the peripheral blood vessels (Lizanecz et al. 2006). Later research on the effects of TRPV1 stimulation on the blood vessels suggested both dilation and constriction upon TRPV1 stimulation (Kark et al. 2008). TRPV1 mediated dilations were related to the perivascular sensory neuronal terminals, which may release neurotransmitters (CGRP, substance P) upon stimulation, mediating vasodilation (Zygmunt et al. 1999). The vasoconstrictive TRPV1 was much less characterised. Nonetheless, we (Czikora et al.

2012; Kark et al. 2008) and others (Cavanaugh et al. 2011) have recently proven that functional TRPV1 is expressed in arteriolar smooth muscle cells, where its activation result is an increase in intracellular  $\text{Ca}^{2+}$  concentration and vasoconstriction.

Transient receptor potential vanilloid (TRPV1-6) channels are gaining an attention in vascular biology. They are cation channels, with some selectivity to  $\text{Ca}^{2+}$  (Baylie and Brayden 2011). However, there is little consistency in the reports regarding the role of TRPV1 in vascular biology. In particular, some of the reports suggest that the same arteries can respond to capsaicin by dilation and also by constriction depending on the conditions (Baylie and Brayden 2011). The opposite effects of capsaicin on vascular diameter was explained by its localisation in sensory neuronal terminals (mediating dilation) and in vascular smooth muscle cells (Kark et al. 2008).

We performed a detailed study here to reveal functional TRPV1 expression in various vascular tissue of the rat. First, specific antibodies were identified for the sensory neuronal TRPV1 and then vascular TRPV1 expression was studied in detail. These data revealed that TRPV1 expression in the vascular beds are not uniform. Some of the vessels in the same tissue section express TRPV1 while other vessels are apparently without TRPV1 expression. Moreover, TRPV1 responses to capsaicin are greatly different in isolated arteries where TRPV1 appears to be highly expressed, suggesting a tight regulation of TRPV1 sensitivity in arteriolar smooth muscle.

## Methods

### Animals, anaesthesia and general preparation in the *in vivo* experiments

Male Wistar Kyoto (WKY/NCrl) rats (Charles River, Isaszeg, Hungary) were fed ad libitum (chow from Szinbad Kft, Godollo, Hungary). Rats were 250-450 g when experiments were started. Anaesthesia was done by 50 mg/kg i.p. thiopental. Animal experiments were carried out at and approved by the University of Debrecen, Medical and Health Science Center, and were in accordance with the standards established by the National Institutes of Health.

### Total RNA preparation, reverse transcription and RT-qPCR

Tissue samples were prepared as described later for cannulated arteries. Reverse transcription coupled quantitative PCR was performed as described in (Bai et al. 2007). Briefly, total RNA was prepared using Trizol reagent (Life Technologies, Budapest, Hungary) according to the manufacturer's instructions. RNA was treated with DNase and 0.5 µg RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA was purified on Qiaquick PCR cleanup columns (Qiagen, Valencia, CA, USA). 10-fold diluted cDNA was used for qPCR reactions. The quantitative PCR reactions were performed using a LightCycler 480 system (Roche, Basel, Switzerland) and a qPCR supermix (Biocenter, Szeged, Hungary) with the following primers: TRPV1 (fwd: 5'-gaatgacaccatcgctctgc; rev: 5'-

aagagggtcaccagcgtcat) and 36B4 as control (fwd: 5'- ccccgtgtgaggtcacagta; rev: 5'- atgatcagcccgaaggagaa). TRPV1 expression was normalized for 36B4 expression. Finally, the products of the PCR amplification were run on a 2% agarose gel to verify their size. To check for the amplification of primer dimers the template was omitted from the controls and melting curve analysis was performed.

### Immunohistochemistry

Tissue samples (dorsal root ganglia, gracilis muscle, mesenterium, femoral muscle, aorta, carotid artery and the ears) were dissected from the rat and embedded in Tissue-Tek O.C.T compound (Electron Microscopy Sciences, Hatfield, PA, USA). Cryostat sections (thickness 10 µm) were made. Sections were fixed in acetone for 10 min and blocked with normal goat sera (1.5 % in PBS, Sigma, St. Louis, MO, USA, 20 min). TRPV1 was stained by anti-capsaicin receptor antibodies. Antibodies were obtained from Alomone (anti-TRPV1-C, 3<sup>rd</sup> loop) Calbiochem (anti-TRPV1-N), Osenses (3<sup>rd</sup> loop, 4<sup>th</sup> loop) and Neuromics (N-terminal). Details of the source and dilution of the antibodies are shown in Table 1. Blocking peptides (synthesized based on the immunogenic TRPV1 fragment used to develop the antibodies) were also used in some cases. Binding of the TRPV1 specific antibodies was visualized by fluorescent secondary antibodies (Table 1) by a Zeiss Meta confocal microscope (Zeiss, Oberkochen, Germany). Tissue sections were also co-stained with an anti-smooth muscle actin (Table 1) or with a neurofilament-

specific antibody (Table 1) in the blocking buffer. Pictures were processed by ImageJ software (freeware from [www.nih.gov](http://www.nih.gov)) to calculate cross sectional areas. Cross sectional areas were calculated by applying the AUTO mode for the dorsal root ganglia pictures in both the Threshold and the Particle analysis menu (the single manual adjustment was to set the maximum area to 1000).

#### Western blot

Tissue samples (dorsal root ganglia and carotid artery) were dissected from the rat. Tissue samples (about 20 ganglia and the two carotid arteries) were pooled, and homogenized in 200  $\mu$ l of SDS sample buffer (S3401, Sigma, St. Louis, MO, USA) and homogenized by a glass tissue homogenizer. Cultured human embryonic kidney cells (HEK293, LGC standards, Wesel, Germany) were transfected by a human TRPV1-expression plasmid (pdEYFP-C1 construct, RZPD, Berlin, Germany). HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (all from Life Technologies, Budapest, Hungary) at 37°C, 5% CO<sub>2</sub>. HEK293 cells (control and transfected) were collected from 100 mm diameter Petri Dishes and homogenized in 500  $\mu$ l of SDS sample buffer. All homogenized samples were incubated at 100°C for 10 min. Protein concentration was determined using a BSA standard. 30  $\mu$ g of protein was loaded onto 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were then stained by a reversible protein staining dye (Ponceau S) and were

cut into strips each containing the above mentioned samples and a prestained molecular weight standard (ProSieve QuadColor, Lonza, Rockland, Maine, USA) and probed by TRPV1 specific antibodies (Table 1). Blocking peptides (Table 1) were obtained from Alomone, (Jerusalem, Israel, ACC-030 antibody) and from EZBiolab (Caramel, IN, USA, PC547 antibody). Blocking peptides were used in a ratio of 1 µg peptide : 1 µg antibody and were preincubated with their respective antibodies for 60 min at room temperature. All primary antibodies (Table 1) were suggested to work in Western blotting by the company data sheets. The dilutions suggested by the manufacturers were used here (Table 1). Binding of the primary antibodies were detected by an anti-rabbit-POD secondary antibody (Sigma, St. Louis, MO, USA). Peroxidase reaction was detected by ECL (Western lighting plus ECL, PerkinElmer, Waltham, MA, USA) and the signal was recorded by an imaging system (MF-Chemibis 3.2, Central European Biosystems, Budapest, Hungary). All of the membranes showed on Fig. 5 were developed together, exposition time was 6 min. Intensity range on the pictures shown on Fig. 5 is 0-15984 (recording was done in the range of 0-65535).

#### Preparation of cannulated arterioles

Isolation of the skeletal (gracilis) muscle arterioles of the rat and diameter measurement of the arterioles were performed as described earlier (Lizanecz et al. 2006). Preparation of small mesenteric resistance arteries were performed likewise. The internal diameter of the arterioles were measured by video microscopy. Experiments were carried out in PSS

(composition in mM: 110 NaCl, 5.0 KCl, 2.5 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 1.0 KH<sub>2</sub>PO<sub>4</sub>, 5.0 glucose and 24.0 NaHCO<sub>3</sub> equilibrated with a gas mixture of 10% O<sub>2</sub> and 5% CO<sub>2</sub>, 85% N<sub>2</sub>, at pH 7.4). After the development of spontaneous myogenic tone at 80 mmHg arteriolar responses to acetylcholine (endothel dependent dilation, 1 nM-10 μM), to norepinephrine (vasoconstrictor, 1 nM-10 μM) and to capsaicin (TRPV1 agonist, 1 nM-10 μM) were measured. Agonists were applied in a cumulative fashion.

#### Measurement of arteriolar contractions under isometric conditions

Large arteries were prepared from the rat in Ca<sup>2+</sup> free PSS. 4 mm long segments of the vessels were fixed on a contractile force measurement setup (DMT510A, Danish Myotechnology, Aarhus, Denmark). After fixing of the vessels on the setup in Ca<sup>2+</sup>-free PSS, the buffer was changed to PSS and vessels were stretched by 10 mN. Mounted arteries were incubated in PSS for 40-60 min (until force values were stabilized) at 37°C. Experiments were started by the addition of the smooth muscle dependent vasoconstrictive agent norepinephrine or U-46619 in a cumulative fashion (1 nM-10 μM). After reaching the maximal response, these agents were washed away and the vessels were incubated in PSS alone, until the contractile force decreased to the level before the application of the vasoconstrictive agents. Finally, capsaicin was applied (1 nM-30 μM) to investigate TRPV1 mediated vascular effects.

#### Materials and solutions

Chemicals were from Sigma-Aldrich (St. Louis, MO, USA) if not stated otherwise.

Capsaicin (8-methyl-N-vanillyl-*trans*-6-nonenamide) was dissolved in ethanol.

Norepinephrine and acetylcholine were dissolved in distilled water.

#### Data analysis and statistical procedures

Arteriolar diameter was determined by measuring the distance between the intraluminal sides of the

arteriolar wall (inner diameter). Data are shown as average diameter  $\pm$  S.E.M. Statistical analysis was made by Microsoft Excel using Student's t-tests. P-values  $<0.05$  were considered to be significant.

## Results

### *Non-neuronal expression of TRPV1*

The expression of TRPV1 in vascular tissues were tested by RT-PCR (Fig. 1A) and Q-PCR (Fig. 1B) in isolated vascular preparations. TRPV1 expression was found to be the highest in dorsal root ganglia. Nonetheless, TRPV1 was also expressed in other tissues, albeit its expression was two orders of magnitude lower than that of in dorsal root ganglia. TRPV1 mRNA expression was not detected in the isolated mesenteric artery (values were similar to those performed without template).

### *Characterization of antibodies developed against TRPV1*

A set of 6 antibodies developed against TRPV1 (Table 1) were tested on dorsal root ganglia of the rat. Two antibodies (anti-TRPV1-N and anti-TRPV1-C) stained specifically a subset of the neurons within the dorsal root among the six tested, while three antibodies (Alomone 3<sup>rd</sup> loop, Osenses 3<sup>rd</sup> loop and Osenses 4<sup>th</sup> loop) did not give any cell specific staining pattern and the Neuromics N-terminal antibody had a rather nonspecific neuron staining pattern under these conditions (Fig. 2). The anti-TRPV1-N and anti-TRPV1-C antibodies were tested in detail. Both the anti-TRPV1-N (Fig. 3A) and anti-TRPV1-C (Fig 3B) antibodies stained (red color on the figure) a subset of cell bodies within the dorsal root ganglia of the rat. TRPV1 positive cells were also stained by a neurofilament specific antibody, although the intensity of the signal (green color on Fig. 3) was weaker in the case of TRPV1 expressing cells than that for the cells which did not

show TRPV1 positivity. Images recorded at higher magnification in separate experiments confirmed this observation (Fig. 4A and Fig. 4C). TRPV1 specific immunostaining disappeared when the anti-TRPV1 antibodies were pre-absorbed with their respective blocking peptides (anti-TRPV1-N, Fig. 4B, anti-TRPV1-C, Fig. 4D). The company data sheets for the TRPV1 antibodies tested in immunohistochemistry (Fig. 2, Table 1) state that they are suitable for Western blotting. Antibodies were therefore tested in Western blot on dorsal root ganglia, carotid artery, HEK293 and TRPV1-overexpressing HEK293 cell homogenates (Fig. 5). Some of the antibodies (Alomone 3<sup>rd</sup> loop, Osenses 4<sup>th</sup> loop and Neuromics N-terminal, Fig. 5A-5C) gave only nonspecific signals (Fig. 5). The anti-TRPV1-N antibody did not give any signal under these conditions (Fig. 5E) over the background (Fig. 5D). Nonetheless, the anti-TRPV1-C antibody (Fig. 5F) was found to be applicable in Western blotting. In particular, it detected TRPV1 in dorsal root ganglia and in TRPV1-overexpressing HEK293 cells (but not in untransfected HEK293 cells). Moreover, this TRPV1 specific signal disappeared in the presence of the blocking peptide.

#### *Characterization of TRPV1 positive structures in the dorsal root ganglia*

The anti-TRPV1-N and anti-TRPV1-C antibodies were used to investigate TRPV1 expression in the rat. The cross sectional area of the TRPV1 positive neurons was measured in the dorsal root ganglia (anti-TRPV1-N, Fig. 6A and anti-TRPV1-C, Fig. 6B). Both antibodies stained the small diameter neurons (Fig. 6E and 6F, respectively,

cross sectional diameter was 200-600  $\mu\text{m}^2$ ), which was in accordance with the size of the sensory neurons. Surprisingly, the anti-TRPV1-N antibody also gave a TRPV1-like immunoreactivity in vascular beds within (Fig. 6A) or around (Fig. 3A) of the dorsal root ganglia. To this end colocalization of TRPV1 with smooth muscle actin was tested. The anti-TRPV1-N antibody stained some, but not all smooth muscle cells (Fig. 6B). It appeared that some vessels express TRPV1, while other vessels are not in the same tissue section. The anti-TRPV1-C antibody did not stain smooth muscle cells (Fig. 6D). Functional expression of TRPV1 in the sensory neurons is well established, but its expression in the vasculature is a relatively novel concept. The goal of the present study was to investigate this vascular TRPV1 by a combination of immunohistochemistry and functional measurements.

*Characterization of functional TRPV1 expression in different vascular tissues of the rat*

Vascular smooth muscle cells of blood vessels within the gracilis muscle of the rat were positively stained by an anti-TRPV1-N antibody (Fig. 7B), while the anti-TRPV1-C antibody did not produce a specific staining pattern (Fig. 7D). None of these antibodies stained neurites in this tissue (Fig. 7A and 7C, respectively). The TRPV1 positive (anti-TRPV1-N antibody) arteries were isolated and the effects of a TRPV1 agonist capsaicin was tested. Capsaicin evoked a robust constriction in these arterioles, which was comparable to that evoked by norepinephrine (Fig. 7E).

These conflicting staining patterns of vascular tissue by the two TRPV1 antibodies were further investigated by the application of the blocking peptides. The smooth muscle staining by the anti-TRPV1-N antibody (Fig. 8A) was blocked by the immunogenic TRPV1 fragment (Fig. 8B), confirming the TRPV1 specificity of the staining. On the other hand, there was no signal above the background in the case of the anti-TRPV1-C antibody (Fig. 8C and 8D).

An inhomogeneous staining pattern was found in the mesenteric tissue with the anti-TRPV1-N antibody (Fig. 9A and 9B), while the anti-TRPV1-C antibody (Fig. 9C and 9D) was again without specific staining. Some of the blood vessels were positive for TRPV1, while some other vessels were not stained in the same tissue section (Fig. 9A and 9B). Capsaicin was without functional effects, although norepinephrine evoked substantial vasoconstrictions (Fig. 9E).

The anti-TRPV1-N antibody gave a strong positive staining in case of the femoral artery (Fig. 10B), while the anti-TRPV1-C antibody showed a weak background staining in the skeletal muscle cells (Fig. 10D). Capsaicin was without effects in the functional measurements on these (isolated) arteries, compared to the constrictions evoked by norepinephrine (Fig. 10E). None of the peripheral neurites were stained by these antibodies (Fig. 10A and 10C).

The next vascular tissue to be studied was the aorta. The aorta was positively stained for TRPV1 by the anti-TRPV1-N antibody (Fig. 11B), but not with the anti-TRPV1-C antibody (Fig. 11D). Capsaicin was without effects on the isolated rings, while

norepinephrine evoked substantial constrictions (Fig. 11E). There was no neuronal staining in these tissue sections (Fig. 11A and 11C).

Carotid artery was also tested. Again, the anti-TRPV1-N antibody stained the smooth muscle layer of the tissue (Fig. 12B), while the anti-TRPV1-C antibody was without specific staining (Fig. 12D). Nonetheless, capsaicin evoked a partial constriction in carotid arteries, which was about 20% of the maximal constriction, achieved by the thromboxane A2 agonist U-46619 (Fig. 12E). Besides to the vessels, no neuron specific staining was found (Fig. 12A and 12C).

Finally, a tissue important in the thermoregulation was tested. Staining of the ears of the rat revealed that both the anti-TRPV1-N and anti-TRPV1-C antibodies can stain peripheral axons (Fig. 13A and 13C, respectively). The anti-TRPV1-C antibody did not stain specifically other structures (Fig. 13D). In contrast, the anti-TRPV1-N antibody again stained some of the blood vessels (Fig. 13B). It is important to note, that a very limited number of blood vessels were stained, and they appeared to have a larger diameter (Fig. 13B).

## Discussion

There are numerous reports about TRPV1 expression in non-neuronal tissues. Here we made an effort to characterize these TRPV1 populations in the peripheral tissues of the rat. The presence of TRPV1 mRNA was established first. Q-PCR data (Fig. 1B) suggested, that TRPV1 is indeed expressed in vascular preparations, although the expression level was about two orders of magnitude lower than that is in dorsal root ganglia.

After establishing the presence of TRPV1 in vascular tissues an effort was made to identify antibodies suitable for the detection of TRPV1 expression. An extensive testing of six commercially available antibodies was performed. It was found that only two of the 6 tested antibodies stained TRPV1 selectively in the dorsal root ganglia of the rat. These antibodies (referred as anti-TRPV1-N and anti-TRPV1-C) were then characterized in detail. It was found that one of the antibodies tested (anti-TRPV1-C) detects endogenous (dorsal root ganglia) and exogenous (transfected HEK293 cells) TRPV1 in Western blotting, which can be blocked by the antigenic peptide. Moreover, TRPV1 staining of sensory neurons (anti-TRPV1-N and anti-TRPV1-C antibodies) and vascular smooth muscle cells (anti-TRPV1-N antibody) was blocked by the antigenic peptides, suggesting that these antibodies are specific for TRPV1.

We have concentrated our efforts to the blood vessels and the staining was performed in parallel with functional measurements on the same arteries, when it was possible. First, the gracilis muscle was tested, because in the case of skeletal muscle

arteries we have previously provided a convincing set of data suggesting functional TRPV1 expression (Czikora et al. 2012; Kark et al. 2008; Lizanecz et al. 2006). Indeed, TRPV1 was found to be abundantly expressed in all blood vessels within the gracilis muscle by the anti-TRPV1-N antibody. Interestingly, the anti-TRPV1-C antibody did not give any positive staining in the case of this tissue suggesting that the anti-TRPV1-C antibody does not recognize the vascular smooth muscle located TRPV1, while it is suitable for the detection of TRPV1 in sensory neurons by Western blot and immunohistochemistry. One may argue, that the vascular smooth muscle staining observed by the anti-TRPV1-N antibody is an artifact, but there are many reasons making this hypothesis unlikely. In particular, (1) vascular TRPV1 staining was blocked by the TRPV1 specific antigenic peptide (Fig. 8); (2) the vascular TRPV1 expression is in accordance with the constrictive effect of the TRPV1 agonist capsaicin. Capsaicin mediated vasoconstriction is missing in TRPV1<sup>-/-</sup> mice (Czikora et al. 2012), strongly suggesting that capsaicin response is specific for TRPV1; (3) TRPV1 mRNA is present in the isolated arteriolar preparations (Fig. 1); (5) earlier reports by an independent group also showed functional arteriolar TRPV1 expression (Cavanaugh et al. 2011).

The goal of the present work was to study TRPV1 expression and function in a set of rat tissue samples and on isolated arteries, using the anti-TRPV1-C antibody as a TRPV1 expression marker in the vascular tissue. There were several important observations. First, it turned out, that the TRPV1 is not uniformly expressed in the vascular tissues. It was found that TRPV1 only expressed in a subset of blood vessels in

some tissues (in particular mesenteric, and skin tissues). The observed differences in TRPV1 stainings (in the same tissue sections) suggests a complex regulation of TRPV1 expression at the level of the individual vessels.

Another surprising observation was that the functional responses of the TRPV1 positive (anti-TRPV1-N antibody) arteries were in a wide range. While arteries from the gracilis muscle responded to capsaicin by a robust constriction, which was comparable to that of evoked by norepinephrine (representing the maximal physiological vasoconstriction in this particular case), other arteries (e.g. carotid artery) had a limited functional TRPV1 response even if an apparently high level of TRPV1 expression was found. Moreover, some of the arteries expressing TRPV1 did not contracted upon capsaicin application (femoral and mesenteric arteries, aorta).

These apparent controversies between TRPV1 function and expression may be explained by many factors. First, it is possible that TRPV1 expression is regulated at the cellular level. One of the observations, supporting this hypothesis is that only a portion of isolated coronary smooth muscle cells responded to capsaicin by increasing intracellular  $Ca^{2+}$  concentrations (Czikora et al. 2012). Another possibility is that TRPV1 expression is regulated at the level of the individual blood vessels (Cavanaugh et al. 2011). It is supported by the staining patterns here, showing that the smooth muscle layer is uniformly stained in a given blood vessel, although some of the blood vessels are stained while others are not in the same tissue slice. It needs to be mentioned, that this vessel specific staining pattern has also been reported earlier with a genetically engineered mice

(genetical labelling of TRPV1 by a fluorescent protein), although the same group was not able to stain unmodified TRPV1 in the same localization (Cavanaugh et al. 2011). Finally, it is also possible that activity of vascular TRPV1 is also controlled by posttranslational modifications. In this case the physiological activity of vascular TRPV1 may be regulated by sensitization-desensitization of the receptor. As a matter of fact, it was found that vascular TRPV1 can be desensitized, which process involved the activation of the  $\text{Ca}^{2+}$  dependent phosphatase, calcineurin (Lizanecz et al. 2006).

An alternative explanation for the differences in the arteriolar response to capsaicin in case of gracilis, femoral, carotid arteries and aorta may be the existence of different TRPV1 isoforms. All the preparations gave strong staining with the anti-TRPV1-N antibody. Nonetheless, at least three different TRPV1 splicing variants have been suggested, which has low sensitivity for agonists (such as the capsaicin used here) (Eilers et al. 2007; Tian et al. 2006; Vos et al. 2006; Wang et al. 2004). Moreover, these splice variants may associate with the dominant (functional) form of the TRPV1, and inhibit its activity (without affecting its apparent expression when tested by immunohistochemistry). Here we performed a morphological study, in which we found striking differences in the TRPV1 staining pattern within arteries in close proximity (on the same tissue section) and showed that large arteries with apparently similar TRPV1 expression have strikingly different capsaicin sensitivity. It requires further studies to identify the reasons of these differences. Regardless of the complicated regulation, the main question is the functional role of TRPV1 in vascular biology. TRPV1 mediated

vasoconstriction was reported three decades ago. In particular, Donnerer and Lembeck described that capsaicin evokes a three-phase response in the rat. The second phase of this response was a vasoconstrictive response, which was found to be independent of sensory innervation (Donnerer and Lembeck 1982). Duckles even described the direct effect of capsaicin on vascular smooth muscle cells (Duckles 1986). The presence of TRPV1 in vascular tissue was further confirmed after the identification of its gene. Recently, functional TRPV1 expression in the smooth muscle cells of intrapulmonary arteries was related to pulmonary hypertension (Martin et al. 2012). Moreover, TRPV1 staining in the smooth muscle layer of epineurial arteries together with a contractile response to capsaicin and resiniferatoxin was also noted previously (Davidson et al. 2006). Our data confirm these earlier observations. In addition to the confirmation of earlier results, we were able to show that arteriolar TRPV1 expression is tightly regulated (arteries within a couple of micrometers show high or undetectable level of TRPV1 expression) and that TRPV1 responses are suppressed in some cases, in spite of uniformly high TRPV1 expression.

These data provides evidence that TRPV1 represents an other significant vascular TRP channel. Our data suggest that TRPV1 is in its sensitized state in some skeletal muscle arteries (such as isolated from the gracilis muscle as presented here) and in the coronary arteries (Czikora et al. 2012), but it is inhibited in other blood vessels. It is also known, that TRPV1 stimulation has a divergent effects *in vivo*: it may evoke neurogenic vasodilation (for example in the skin) and it may also evoke vascular constrictions (in the

skeletal muscle) (Kark et al. 2008). These divergent effects suggest that systemic regulation of TRPV1 may be an effective tool to regulate blood distribution between tissues. Moreover, these divergent effects are in accordance with the immunohistochemical data presented here. It is expected that arteries with sensory neuronal innervation, but without vascular TRPV1 expression (such as blood vessels in the skin, Fig. 13) respond to TRPV1 stimulation by dilation. In contrast, arteries with high smooth muscle TRPV1 expression and without apparent sensory neuronal innervation (such as gracilis artery, Fig. 8) are expected to respond to the same TRPV1 activation by constriction. An important addition to this hypothesis by this work is that it appears that TRPV1 expressed in the arteries is not necessarily functionally active. As a matter of fact, we have shown here that although TRPV1 is expressed in the large arteries, the activity of this receptor appears to be suppressed.

Taken together here we made an effort to investigate the functional expression of TRPV1 in the rat. It was found that TRPV1 expression in the vascular smooth muscle is regulated at the level of the individual blood vessels: some blood vessels showed intensive TRPV1 immunostaining, while nearby vessels were negative. Moreover, the activity of the smooth muscle expressed TRPV1 appeared to be suppressed in some cases. Nonetheless, TRPV1 was found to be widely expressed in the vasculature rat, suggesting a physiological role for these cation channels in vascular biology.

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## Tables

Table 1 Details of the antibodies used in the study

Specificity	Region	Developed in	Working dilution	
			Western blot	Immunohistochemistry
TRPV1	C-terminal	Rabbit	1:200	1:500
TRPV1	N-terminal	Rabbit	1:200	1:750
TRPV1	3rd loop	Rabbit	1:200	1:750
TRPV1	3rd loop	Rabbit	N/A	1:100
TRPV1	4th loop	Rabbit	1:200	1:150
TRPV1	N-term	Rabbit	1:1000	1:500
Smooth muscle actin		Mouse	N/A	1:50
Neurofilament		Mouse	N/A	1:100
anti rabbit-biotin		Goat	N/A	1:200
anti mouse-FITC		Goat	N/A	1:200
Streptavidin-Cy3		N/A	N/A	1:500
anti-rabbit-POD			1:40000	N/A

Figure legends

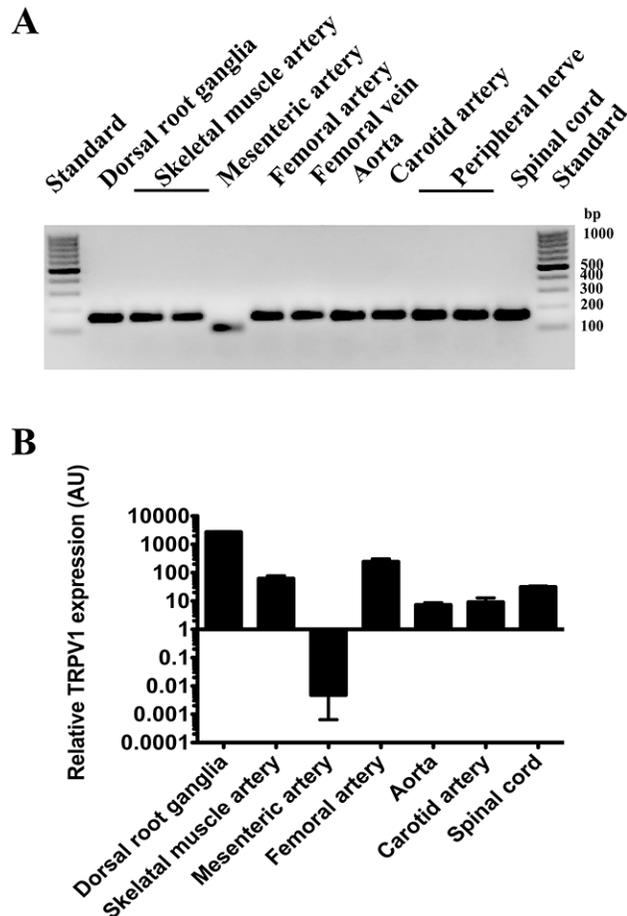


Figure 1 TRPV1 mRNA in peripheral tissues of the rat

TRPV1 expression was tested as the mean of RT-PCR (A) and Q-PCR (B) in peripheral tissues of the rat. Isolated mRNA (0.5  $\mu$ g) from various tissue sources (isolated arteries, veins, nerves, dorsal root ganglia and spinal cord) was subjected to RT-PCR and Q-PCR with a primer set specific to the rat TRPV1. Reaction mixtures were loaded on 2% agarose gels to separate PCR products. Bands at the apparent molecular size of 170 bp

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were in accordance with the expected size of the product, while the band in the case of the isolated mesenteric artery was aspecific. Q-PCR experiments (B) revealed negligible expression of TRPV1 in mesenteric arteries (values were similar to those performed without template), but a reasonably high level of expression was found in other peripheral tissues (n=2-8, bars represent mean±SEM).

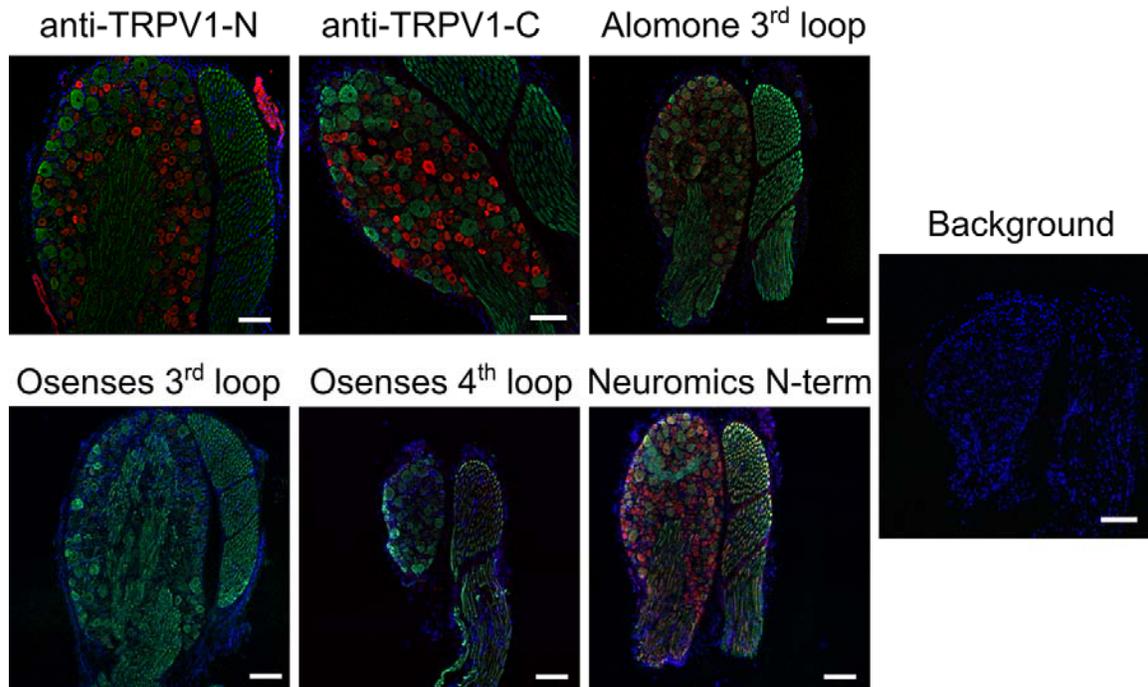


Figure 2 Specificity of TRPV1 antibodies

Six commercially available anti TRPV1 antibodies were tested on dorsal root ganglia (cryostat sections) of the rat (red). Tissue sections were co-stained by a neurofilament specific antibody (green, neurons). Background staining was obtained by omitting the primary antibodies. Primary antibodies are indicated on the figure. Bars represent 100  $\mu\text{m}$ . Dilutions and details of the antibodies are summarized in Table 1.

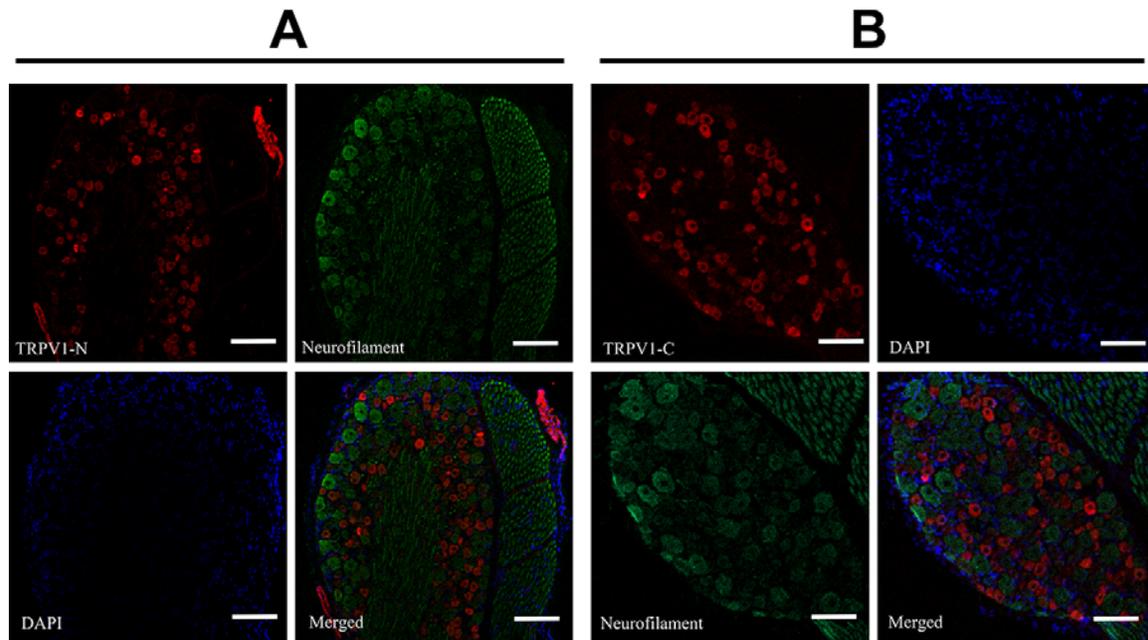


Figure 3 Colocalization of TRPV1 and neurofilament immunoreactivities

Dorsal root ganglia of the rat was stained by anti-TRPV1-N (A) and anti-TRPV1-C (B) antibodies (red channel) together with a neurofilament specific antibody (green channel) and a DAPI counter stain (blue channel). The merged images are also shown, besides to the separate channels. Bars represent 100  $\mu\text{m}$ .

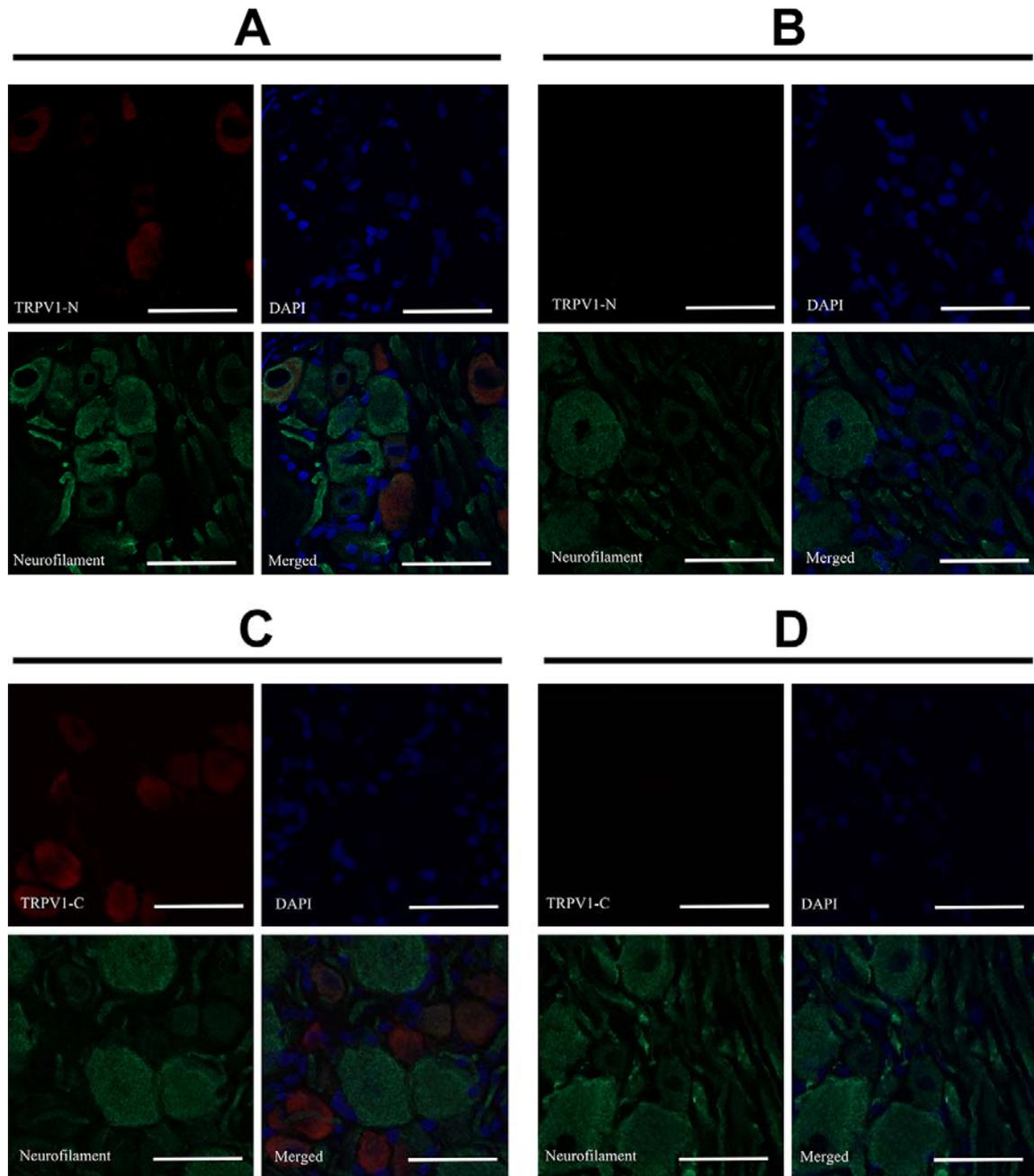


Figure 4 Specificity of neuronal TRPV1 staining

Dorsal root ganglia of the rat was stained by anti-TRPV1-N (A and B) and anti-TRPV1-C (C and D) antibodies (red channel), together with a neurofilament (green channel) and

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DNA (DAPI, blue channel) markers. Blocking peptides were synthesized according to the sequence of the immunogenic TRPV1 fragment to investigate the specificity of the TRPV1 staining. The positive staining disappeared when the antibodies were pre-incubated with the blocking peptides (B and D). Bars represent 50  $\mu$ m.

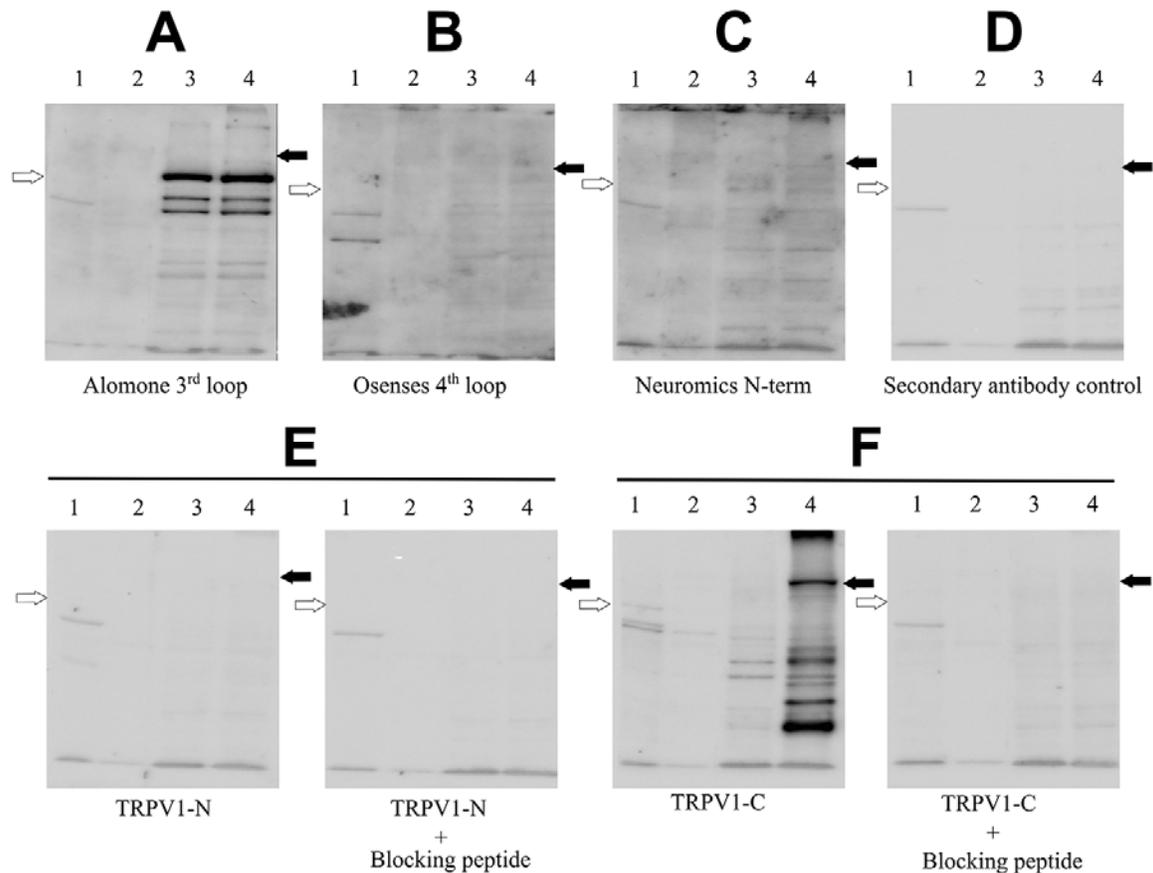


Figure 5 Specificity of TRPV1 antibodies in Western blotting

Dorsal root ganglia (1) and carotid artery (2) was prepared from the rat. HEK293 (3) and HEK293 cells transfected by a GFP-TRPV1 encoding plasmid (4) were cultured in cell culture dishes. Samples were prepared in SDS sample buffer and loaded on 10% polyacrylamide gels (30  $\mu$ g protein/well). Separated proteins were transferred onto nitrocellulose membranes and probed by antibodies developed against TRPV1. The dilutions were set as the product booklets suggested or at 1:50 (anti-TRPV1-N antibody). Membranes were incubated simultaneously with the secondary antibody (goat anti rabbit-POD) and ECL was used for the detection. All of the membranes were developed

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simultaneously in a Chemibis 3.2 imager. Exposure time was 6 min. Expected position of the endogenous TRPV1 is shown by empty arrows, filled arrows represent the position of the GFP-TRPV1.

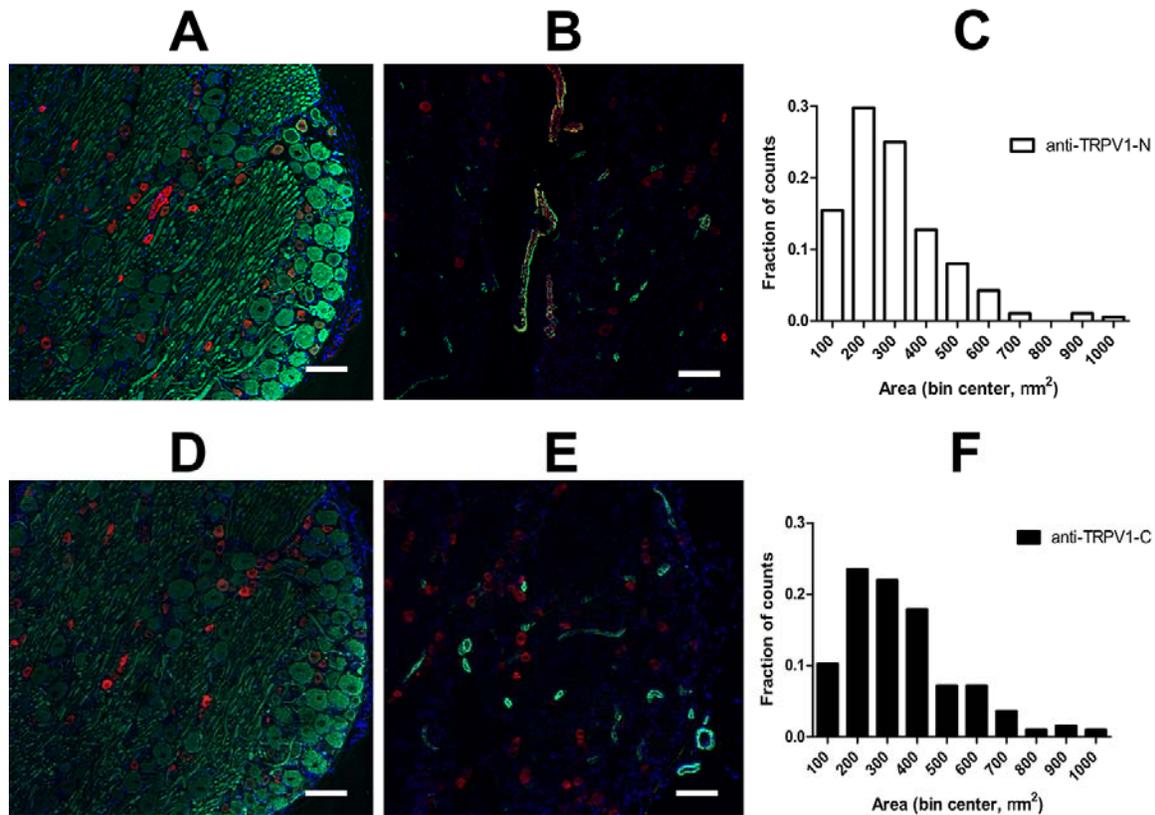


Figure 6 TRPV1 expression in sensory neurons

The selectivity of the anti-TRPV1-N (A and B) and anti-TRPV1-C (C and D) antibodies were tested in the dorsal root ganglia of the rat. Cryostat sections (10  $\mu$ m) were stained by the TRPV1 antibodies (red), by a neurofilament (green, A and C) and a smooth muscle actin (green, B and D) specific antibody. DNA was labelled by DAPI (blue). Cross sectional area of the stained cells were calculated and plotted on histograms (anti-TRPV1-N, panel E, anti-TRPV1-C, panel F). Evaluation was made by the ImageJ software.

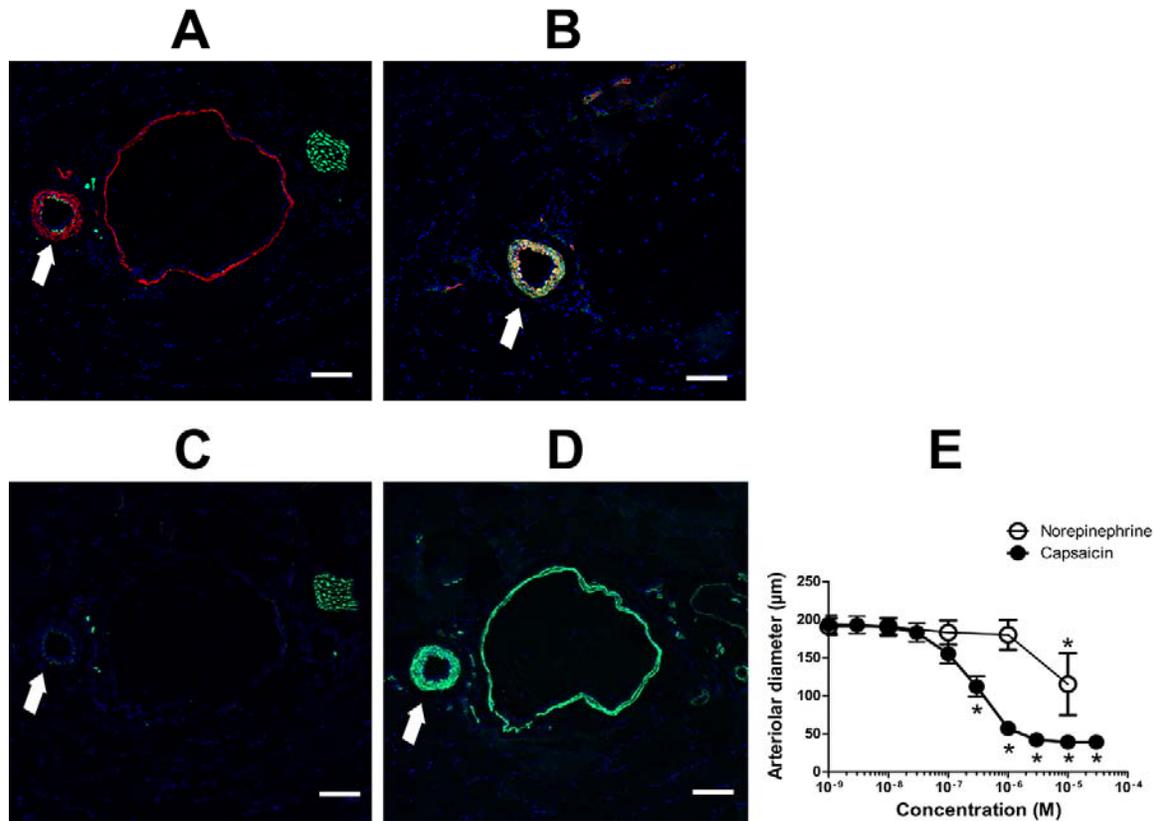


Figure 7 Functional expression of TRPV1 in skeletal muscle blood vessels

Cryostat sections were made from the gracilis muscle of the rat (10 µm) and were stained by anti-TRPV1-N (A and B) and anti-TRPV1-C (C and D) antibodies. TRPV1 (red) was co-stained by neurofilament (green, A and C) and smooth muscle specific actin (green, B and D) specific antibodies. The same arteries (indicated by the empty arrows) were isolated and mounted on an isobaric (cannulated) setup. Concentration-response to capsaicin (a TRPV1 specific agonist) and to norepinephrine are shown on the graph. Symbols represent the mean, error bars are S.E.M. of 5 independent determinations. Significant differences from the initial (before treatment) values are shown by asterisks.

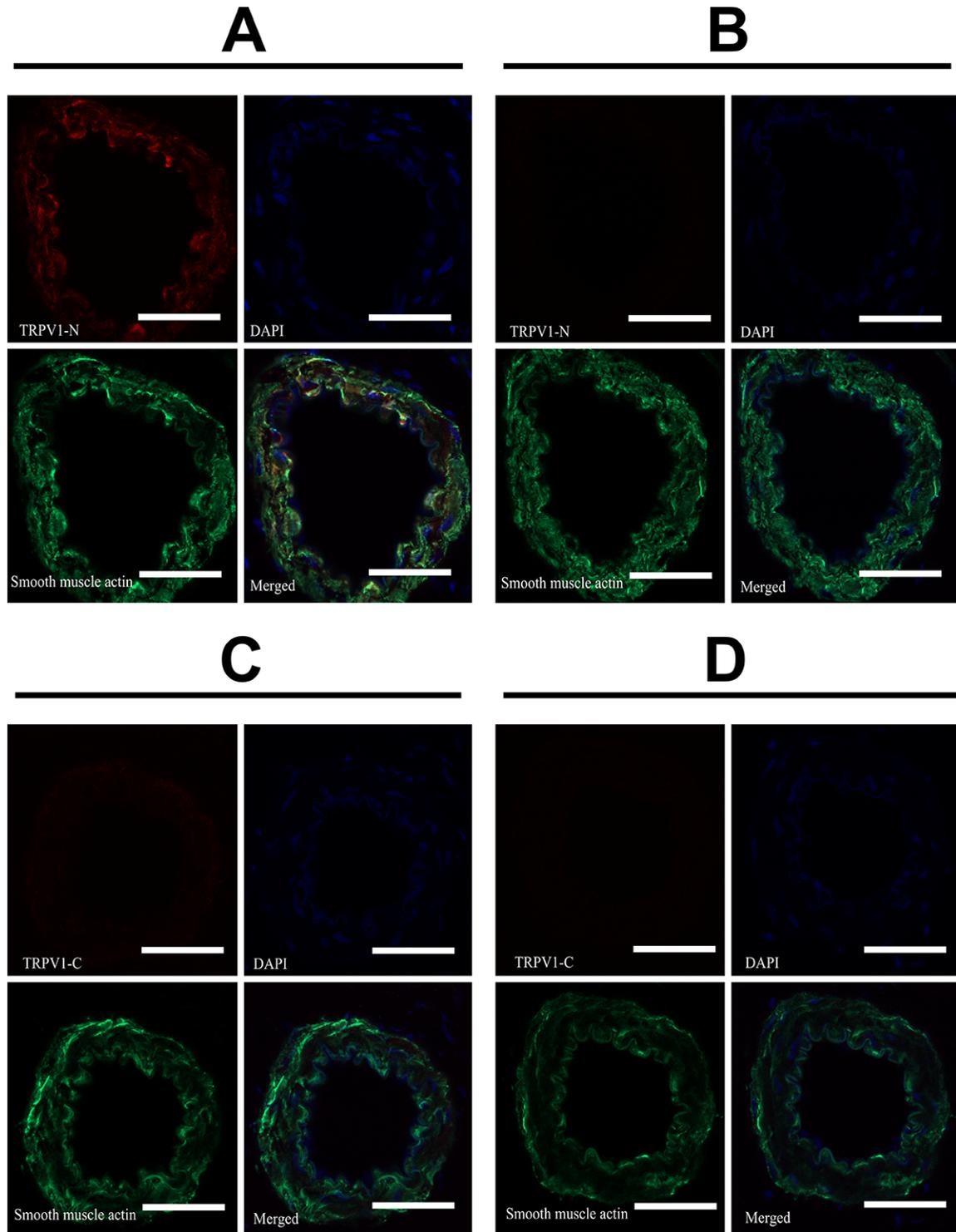


Figure 8 TRPV1 expression in smooth muscle cells

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The smooth muscle expression of TRPV1 was investigated in detail. Gracilis muscle tissue sections of the rat were probed with anti-TRPV1-N (A and B, red channels), anti-TRPV1-C (C and D, red channels), anti-neurofilament (green channels) antibodies and with DAPI (blue channels). Merged images are also shown. In some cases the TRPV1 specific antibodies were pre-absorbed by blocking peptides (preincubation with the blocking peptides, B and D). Note missing red staining in these cases, in particular on Panel A (colocalization of TRPV1 with the smooth muscle actin) versus Panel B (blocked TRPV1 binding, maintained smooth muscle actin staining). Bars represent 100  $\mu\text{m}$ .

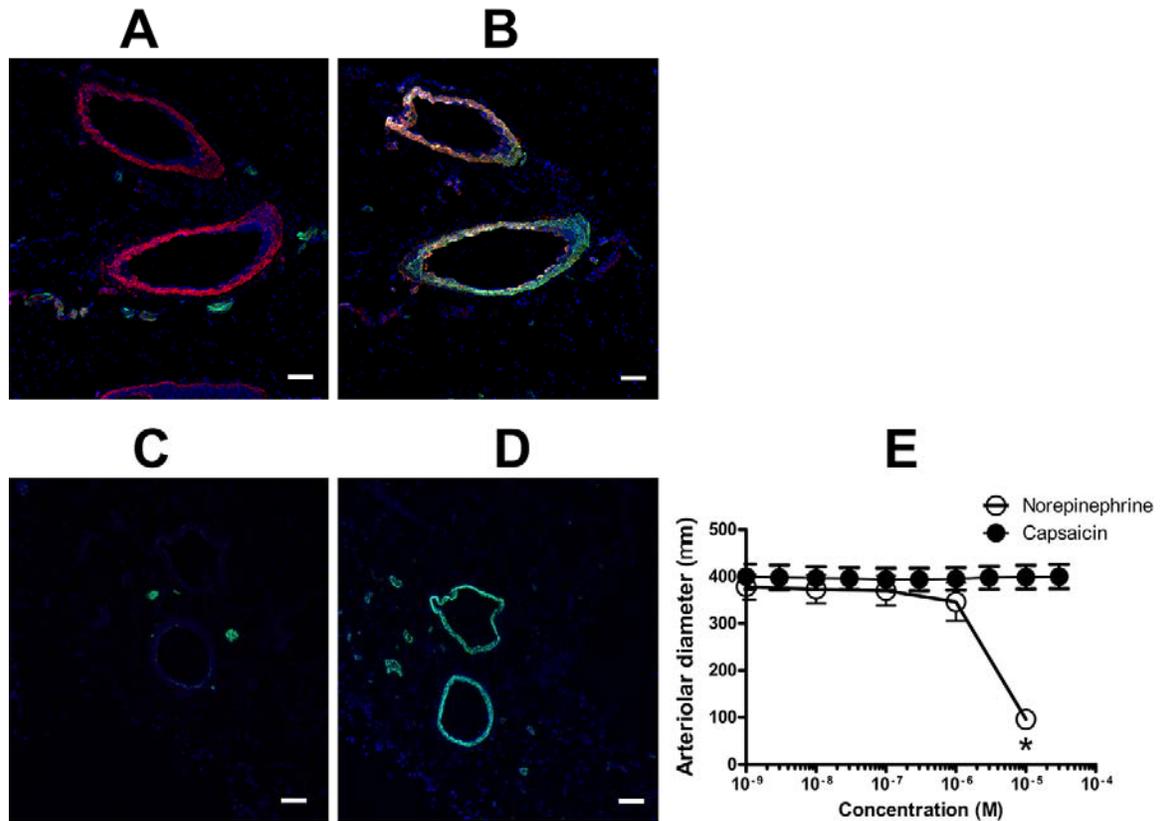


Figure 9 Expression of TRPV1 in mesenteric blood vessels

Cryostat sections were made from the mesenteric tissue (near the small intestines) of the rat (10  $\mu$ m) and were stained by the anti-TRPV1-N (A and B) and anti-TRPV1-C (C and D) antibodies to detect TRPV1 expression. TRPV1 (red) was co-stained by neurofilament (green, A and C) and smooth muscle specific actin (green, B and D) specific antibodies. Isolated arteries from the same tissue (third order arteries) were mounted on an isobaric vascular setup and the responses to capsaicin and to norepinephrine are shown on the graph (E). Symbols represent the mean, error bars are S.E.M. of 5 independent determinations. Significant difference from the initial value is shown by the asterisk. Bars represent 100  $\mu$ m.

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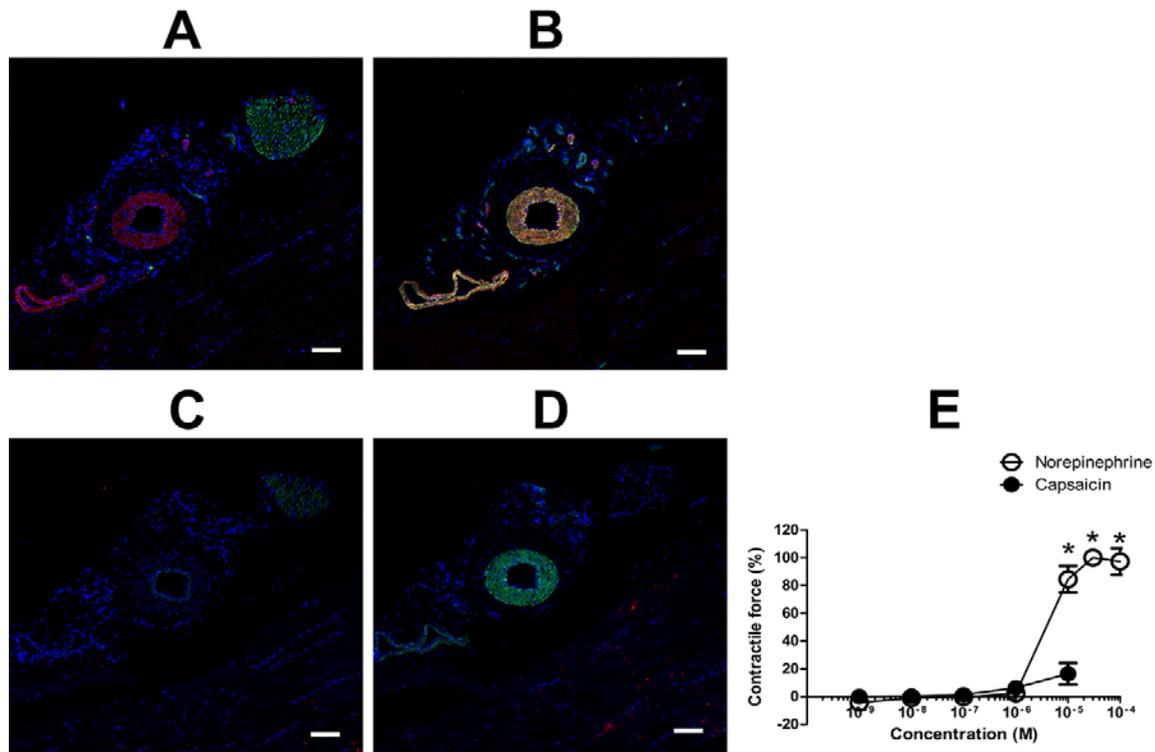


Figure 10 Expression of TRPV1 in the femoral artery

Femoral artery tissue sections were probed by anti-TRPV1-N (red, A and B), anti-TRPV1-C (red, C and D), neurofilament (green, A and C), smooth muscle actin (green, B and D) antibodies and with DAPI (blue channels). Arteries labelled by the empty arrows were mounted on an isometric contractile force measurement system. Responses to capsaicin (TRPV1 specific agonist) and to norepinephrine are shown on the graph (E). Symbols represent the mean, error bars are S.E.M. of 4 independent determinations. Asterisks represent significant constrictions. Bars represent 100  $\mu$ m.

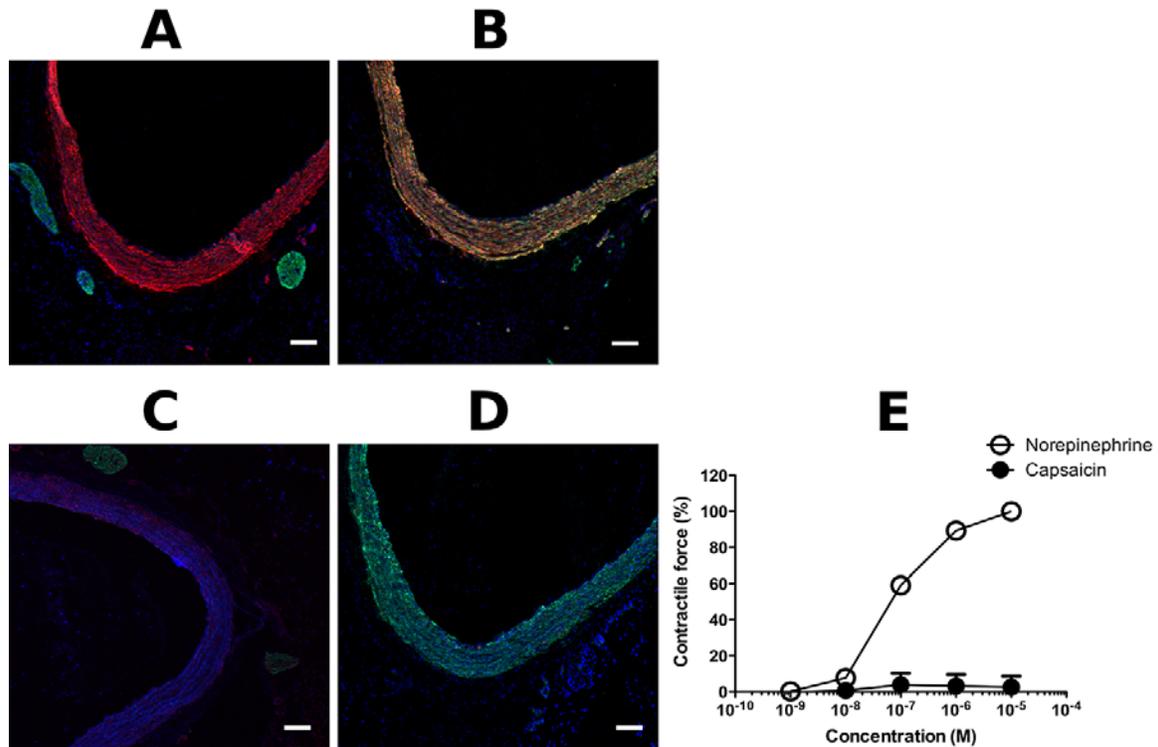


Figure 11 Expression of TRPV1 in the aorta

The aorta of the rat was probed with probed by anti-TRPV1-N (red, Panels A and B), anti-TRPV1-C (red, C and D), neurofilament (green, A and C), smooth muscle actin (green, B and D) antibodies and with DAPI (blue channels). Contractions to capsaicin and norepinephrine were tested in an isometric contractile force measurement system. Results are shown on the graph (E). Symbols represent the mean, error bars are S.E.M. of 6 independent determinations, asterisks show the significant constrictions over the initial values. Bars represent 100  $\mu$ m.

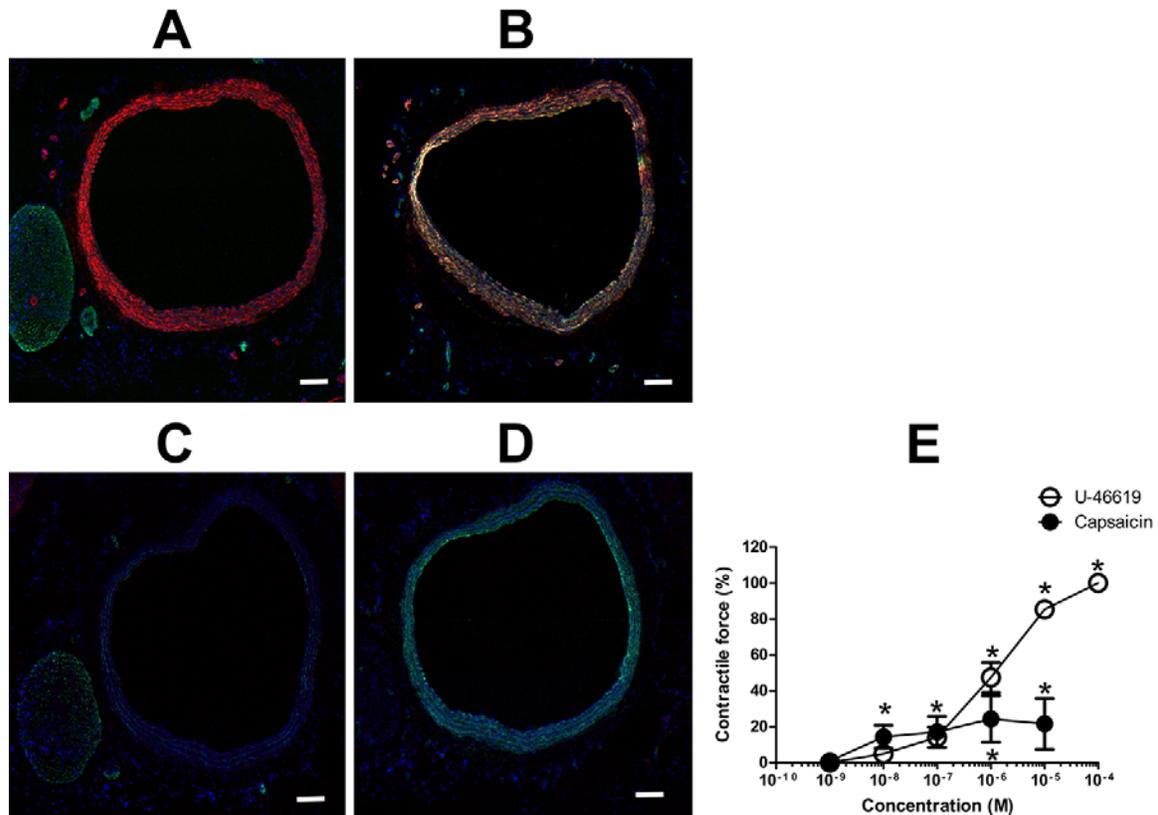


Figure 12 Expression of TRPV1 in the carotid artery

Cryostat sections were made from the carotid artery of the rat (10  $\mu$ m) and were stained by the anti-TRPV1-N (A and B) and anti-TRPV1-C (C and D) antibodies to detect TRPV1 expression. TRPV1 (red) was co-stained by neurofilament (green, A and C) and smooth muscle specific actin (green, B and D) specific antibodies. The same arteries were isolated and mounted on an isometric (ring) setup. Concentration-responses to capsaicin and to U-46619 (a thromboxane A<sub>2</sub> receptor agonist) are shown on the graph (E). Symbols represent the mean, error bars are S.E.M. of 8 independent determinations. Significant differences from the initial contractile force values are shown by the asterisks. Bars represent 100  $\mu$ m.

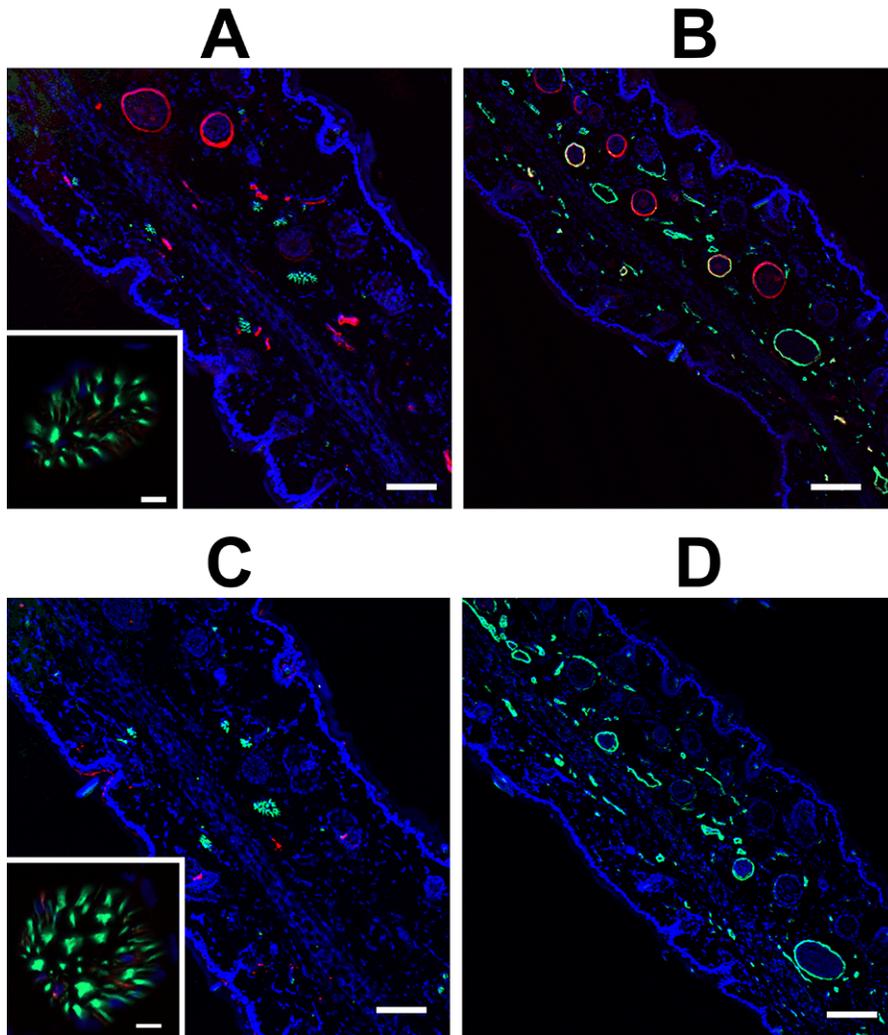


Figure 13 Expression of TRPV1 in the skin

Immunohistochemistry was performed in the skin at the hindpaw of the rat. Anti-TRPV1-N (red, A and B), anti-TRPV1-C (red, C and D), neurofilament (green, A and C), smooth muscle actin (green, B and D) antibodies and with DAPI (blue channels). Colocalization of TRPV1 positive neurites with the neurofilament marker are shown in the inserts by higher magnification (bars within the inserts represent 10  $\mu\text{m}$ ). Bars in the lower magnification images represent 100  $\mu\text{m}$ .