ABSTRACT

Background: Peripheral application of opioids reduces inflammatory pain but is less effective in noninflamed tissue of rats and human patients. Hypertonic solutions can facilitate the antinociceptive activity of hydrophilic opioids in noninflamed tissue in vivo. However, the underlying mechanisms are not well understood. We hypothesized that the enhanced efficacy of opioids may be because of opening of the perineurial barrier formed by tight junction-proteins like claudin-1.

Methods: Male Wistar rats were treated intraplantarly with 10% NaCl. Pain behavior (n = 6) and electrophysiological recordings (n = 9 or more) from skin-nerve preparations after local application of the opioid [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) were explored. Tight junction-proteins as well as permeability of the barrier were examined by immunohistochemistry and Western blot (n = 3 or more).

Results: Local administration of 10% NaCl facilitated increased mechanical nociceptive thresholds in response to DAMGO, penetration of horseradish peroxidase into the nerve, as well as a reduced response of C- but not Aδ-nociceptors to mechanical stimulation after application of DAMGO in the skin-nerve preparation. In noninflamed paw tissue, claudin-1 was expressed in the epidermis, blood vessels, and the perineurium, surrounding neurons immunoreactive for calcitonin gene-related peptide or protein gene product 9.5. Claudin-1 but not claudin-5 or occludin was significantly reduced after pretreatment with 10% NaCl. Intraplantar application of a metalloproteinase inhibitor (GM6001) completely reversed these effects.

Conclusion: Hypertonic saline opens the perineurial barrier via metalloproteinase activation and claudin-1 regulation, thereby allowing access of hydrophilic drugs to peripheral opioid receptors. This principle may be used to specifically target hydrophilic drugs to peripheral neurons.

OPiOIDS can effectively control inflammatory pain both in the central nervous system and peripherally at the site of injury. In inflamed tissue, opioids bind to opioid receptors on peripheral sensory nerve terminals and induce potent analgesia (antinociception) both in humans and in experimental models.1,2 The antinociceptive action of opioids in inflamed tissue is also supported by electrophysiological experiments using skin-nerve preparations.3 In noninflamed tissue, hydrophilic opioids are particularly ineffective, in behavioral, clinical, and electrophysiological studies.3–5 However, intraplantar co-injection of hypertonic solutions with hydrophilic opioids can significantly increase mechanical nociceptive thresholds,4 but the underlying mechanisms are not entirely clear.

The perineurium forms a barrier composed of a basal membrane and a layer of perineurial cells.9 The membranes...
of these cells express proteins for active transport of, e.g.,
glucose, and tight junction proteins to limit paracellular
permeability. Tight junction proteins include the family of clau-
dins and occludins. Earlier studies demonstrated that hyper-
tonic saline increases permeability of the perineurium\textsuperscript{8,9} as well
as the blood-brain barrier. In both barriers several claudins
are expressed. Claudin-1 is a major sealing tight junction
protein. Claudin-1 knockout mice die during the first day of
life because of loss of fluid through the skin.\textsuperscript{10} After nerve
 crush injury, a reduced claudin-1 content in the perineurium
parallels increased permeability to the sciatic nerve.\textsuperscript{9} Previous
studies indicate that metalloproteinases can degrade tight
junction proteins like occludin.\textsuperscript{11–13} Indeed, breakdown of
the blood-brain barrier under pathophysiological conditions
can be ameliorated by treatment with metalloproteinase in-
hibitors, suggesting a role of these enzymes in maintaining
the integrity of the barrier. In this study we examined the
effects of hypertonic saline solutions and hydrophilic opioids
on the time course of peripheral antinociception; electrophys-
iological recordings in an in vitro skin nerve preparation;
penetration of horseradish peroxidase; and the role and reg-
ulation of tight junction molecules in the perineurial barrier.
We hypothesized that opening of the perineurial barrier
formed by tight junction proteins like claudin-1 is respon-
sible for enhanced efficacy of opioids.

Materials and Methods

Animals
Animal protocols were approved by the animal care commit-
tee of the Senate of Berlin (Landesamt für Gesundheit und
Soziales, Berlin) and are in accordance with the International
Association for the Study of Pain.\textsuperscript{14} Male Wistar rats weigh-
ing 180–220 g were injected intraplantarly under brief iso-
flurane anesthesia, as described below. Experiments were
conducted at indicated time-points.

Measurement of Nociceptive Thresholds
Mechanical thresholds were determined using the paw pres-
sure algesiometer (modified Randall-Selitto test; Ugo Basile,
Comerio, Italy), as described before.\textsuperscript{15} The pressure required
to elicit paw withdrawal, the paw pressure threshold (PPT),
was determined by a blinded investigator. Averages from
three measurements per treatment were calculated. Baseline
measurements were obtained before the intraplantar injec-
tion of 100 \( \mu l \) of 0.9% (control) or 10% NaCl under brief
isoflurane anesthesia. In the next step, 3–30 \( \mu m \) DAMGO in
100 \( \mu l \) 0.9% saline were applied either simultaneously or at
indicated time intervals. PPT were determined 10 min there-
after. In some experiments, 2 \( \mu g \) D-Phe-Cys-Tyr-D-Trp-
Orn-Thr-Pen-Thr-NH2 (CTOP; all Sigma-Aldrich Chemical,
St. Louis, MO) or 0.02–0.2 mg GM6001 (United States
Biological, Swamscott, MA) were injected intraplantarly in
100 \( \mu l \) 0.9% saline together with DAMGO. Doses were
chosen based on pilot experiments and literature.\textsuperscript{16,17}

Electrophysiology
In isoflurane-anesthetized rats, 10% NaCl (total volume 300
\( \mu l \)) was injected subcutaneously on the medial aspect of the
foot and medial aspect of the rat lower limb, which is innerv-
ated by the saphenous nerve. After 30 min, an in vitro skin-
nerve preparation was dissected and used to record from
single primary afferents in microdissected filaments of the
saphenous nerve, as described previously. The skin-
nerve preparation was perfused at 15 ml/min with oxygen-
saturated synthetic interstitial fluid buffer containing 123
\( mm \) NaCl, 3.5 \( mm \) KCl, 0.7 \( mm \) MgSO\(_4\), 1.7 \( mm \) NaH\(_2\)PO\(_4\),
2.0 \( mm \) CaCl\(_2\), 9.5 \( mm \) sodium gluconate, 5.5 \( mm \) glucose,
7.5 \( mm \) sucrose, and 10 \( mm \) HEPES at pH 7.4. The mechanical
receptive fields of individual units were identified by
manually probing the skin with a glass rod. A\( \delta \) - and C-fiber
mechanonociceptors were identified by their conduction ve-
locity and threshold to mechanical stimulation by von Frey
hairsticks, as described previously.\textsuperscript{3} Stock solutions of DAMGO
and CTOP were diluted with synthetic interstitial fluid (pH
7.4). 100 \( \mu l \) of oxygen-saturated synthetic interstitial fluid
containing 10 \( \mu M \) DAMGO with or without CTOP was
applied directly to the corium through a small metal ring (10
\( mm \) ID) placed around the receptive field for separation
from the tissue bath. Drugs or vehicle were applied 3 min
before mechanical stimulation. A computer-controlled
nanomotor (Kleindieck, Reutlingen, Germany) was used to
apply controlled displacement stimuli of 10 s duration to the
receptive field at regular intervals (interstimulus period,
60 s).\textsuperscript{18} The probe of the nanomotor was a stainless steel
metal rod and the diameter of the flat circular contact area
was 0.8 mm. The signal driving the movement of the linear
motor and raw electrophysiological data were collected with
a Powerlab 4.0 system (AD Instruments, Spechbach, Ger-
many). Spikes were discriminated off-line with the spike his-
togram extension of the software. All experiments were car-
ried out at an organ bath temperature of 32°C.

Immunofluorescence and Confocal Microscopy
Immunostaining was performed in paw tissue harvested 1 h
after intraplantar injection of 0.9% (control) or 10% NaCl.
In certain experiments hypertonic saline was injected toge-
ther with 0.2 mg GM6001. As a positive control, rats
received only 150 \( \mu l \) complete Freund’s adjuvant (CFA, as
described previously\textsuperscript{16,17}) intraplantarly 2 h before tissue
harvesting.

Tissues were fixed in formalin for 2 h at 4°C, embedded in
diaphanous, and cut into 8-\( \mu m \)-thick cross-sections. Paraffin
was then removed by a xylol-ethanol gradient and sections
were boiled in 1 \( mM \) EDTA or 10 \( mM \) citrate buffer solution.
To block nonspecific staining, tissues were bathed in phos-
phate buffered saline containing 5% (vol/vol) goat serum
(blocking solution) and 1% bovine serum albumin for 60
min at room temperature. All subsequent washing proce-
dures were performed with this blocking solution. For
immunostaining, we used mouse monoclonal anti-occludin
and anti-claudin-1; rabbit anti-occludin and anti-claudin-5 antibodies (1:50, Clones OC-3F10, 2H10D10, Z-T22, and Z43.JK; Invitrogen, San Francisco, CA); guinea pig polyclonal anti-CGRP (1:1000, Peninsula Laboratories, Belmont, CA); and rabbit polyclonal antiprotein gene product 9.5 antibodies (1:100 Dako, Glostrup Denmark) diluted in blocking solution. Tissues were incubated for 60 min and, after two washes, with appropriate secondary fluorescent Alexa Fluor 488 and 594 goat antimouse and goat antirabbit antibodies for 45 min (1:500 Molecular Probes, Hamburg, Germany; MoBiTec). Sections were mounted in ProTags MountFluor (Biocyt, Luckenwalde, Germany). Fluorescence images were obtained with a confocal laser scanning microscope (LSM510Meta, Zeiss, Jena, Germany), using excitation wavelengths of 543 and 488 nm. Concentrations and specific staining in control tissues (gut epithelium19,22) of the antibodies have been shown before by our group.19,23 For quantification of immunofluorescent staining, 12 sections of four animals per group were analyzed, respectively. Black and white images of sections under the specific wave length for claudin-1 staining were obtained with a 40× objective. Perineurial cells in an area of 50 × 50 μm of each section were analyzed densitometrically in a double-blind approach. Images were analyzed densitometrically using AIDA (version 3.53, Raytest Isotopenmessgeraete GmbH; Straubenhardt, Germany). Intensity of specific monochromatic signals was expressed as relative intensity/area.

To assess perineurial permeability rats were treated according to Reference 7. Similar to our previous studies,8 8 mg horseradish peroxidase (HRP) was injected at the indicated time-points after intraplantar injection of 10% saline. After 1 h, rats were perfused with 1.25% glutaraldehyde, 1% paraformaldehyde, 5% sucrose in phosphate buffered saline cated time-points after intraplantar injection of 10% saline.

**Western Blotting**

After indicated treatments, subcutaneous paw tissue was homogenized in lysis buffer for Triton-X100 soluble proteins (25 mM Tris pH 7.6, 120 mM NaCl, 2 mM EDTA, 25 mM NaF, 1% [w/v] Triton × 100) containing protease inhibitors (Complete; Roche Applied Science, Mannheim, Germany).19 Cytosol fractions were obtained by homogenization with minipistil and sonification (3 × 5 s/3-s break), followed by a centrifugation at 200 g for 5 min and subsequent centrifugation of the remaining supernatant at 20,000 g for 60 min. The TX-100-insoluble pellet containing membrane fractions was resuspended in an equal volume of extraction buffer (25 mM Hepes pH 7.6, 2 mM EDTA, 25 mM NaF, 1% [w/v] SDS). Aliquots of protein were mixed with sodium dodecyl sulfate containing buffer, denatured at 95°C for 5 min, fractionated on sodium dodecyl sulfate polyacrylamide gels, and subsequently blotted onto polyvinylidenefluoride membranes (PerkinElmer, Boston, MA). Proteins were detected using an antibody against claudin-1 (Invitrogen) and β-actin as protein loading control (Sigma-Aldrich).19 A specific band in Western blot in control tissues (gut epithelium19) has been shown before by our group. Chemiluminescence signal detection and quantification were done by densitometry (FluorChem FC2 Imaging systems, Multimage II; Alpha Innotech, Santa Clara, CA).

**Statistical Analysis**

Data are presented as mean ± SEM. More than two groups in Western blot and immunohistochemical analyses were compared using ANOVA. The post hoc comparisons were performed by Dunnet’s method compared with control. Repeated measurements in electrophysiological studies were compared by repeated measurements ANOVA with Bonferroni post hoc correction. Multiple measurements with two variables, like in pain behavior studies, were analyzed by repeated measurements two way ANOVA with Bonferroni post hoc correction. Differences were considered significant if P < 0.05. Sigma Stat program (Systat Software, Erkrath, Germany) was used for statistical analysis.

**Results**

**Local Treatment with Hypertonic Saline Facilitates DAMGO-induced Increase in Nociceptive Thresholds Over Extended Periods**

Intraplantar injection of the hydrophilic opioid peptide DAMGO (up to 30 μg) did not change mechanical nociceptive thresholds in paws without inflammation. In contrast, concomitant injection of hypertonic saline permitted a dose-dependent increase in PPT after intraplantar injections of DAMGO (fig. 1A), whereas no difference in baseline PPT was observed (table 1). Contralateral paws were unaffected. The highest dose of DAMGO (30 μg) injected subcutaneously at the animal’s neck did not change mechanical nociceptive thresholds. The injection of hypertonic saline alone did not change these thresholds, either.2 To confirm that the increase in mechanical nociceptive thresholds by DAMGO was dependent on opioid receptors, the selective μ-opioid receptor (MOR) antagonist CTOP was injected concomitantly with DAMGO (30 μg) in rats treated with hypertonic saline intraplantarily (fig. 1B). The effect of DAMGO was fully antagonized by this treatment. To explore the duration of the effect of hypertonic saline, intraplantar DAMGO (30 μg) was injected at different times after 10% saline in separate groups of rats (fig. 1C). PPTs were significantly increased by DAMGO for up to 6 h after a single injection of hypertonic saline. Similar to our previous studies,6 an increase in barrier permeability was observed 10–240 min after
Fig. 1. Long-lasting effects of hypertonic saline solution on DAMGO-induced antinociception. (A) Rats received combined intraplantar injections of different doses of [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) and 100 μl of 10% NaCl (green bars) or 0.9% NaCl (red bars). Contralateral paws were shown as blue bars. Mechanical nociceptive thresholds, such as paw pressure threshold (PPT) were determined 10 min after injection in all experiments (n = 6, two-way repeated-measures ANOVA, Bonferroni post hoc correction, *P < 0.05). (B) Baseline nociceptive thresholds were obtained. Subsequently, animals were intraplantarly injected with 30 μg DAMGO and hypertonic saline in the presence (blue circles) or absence (red circles) of a μ-opioid receptor antagonist (2 μg D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ CTOP) (n = 6, two-way repeated-measures ANOVA, Bonferroni post hoc correction, *P < 0.05). (C) After intraplantar injection of hypertonic saline, five separate groups of rats were reinjected with 30 μg DAMGO intraplantarly at the indicated time-points (n = 6/group, two-way repeated-measures ANOVA, Bonferroni post hoc correction, *P < 0.05). PPT were determined before (baseline, blue circles) and after injection of DAMGO (red squares). The contralateral side is shown for comparison (green triangles). (D) Male Wistar rats (n = 3) were intraplantarly injected with 100 μl 10% NaCl. Control animals did not receive an injection. Staining of horseradish peroxidase from the paw tissue was performed 5–240 min after injection. Intraneural staining was seen at all time points after 10% NaCl (magnification 40×; arrows are pointing at the nerve). A representative example is shown. BL = baseline, TR = treated.
injection of hypertonic saline, as seen by penetration of HRP into the peripheral nerve (fig. 1D).5 No intraneural staining of HRP was observed under control conditions, in line with our previous findings.6

C-fibers but Not δ-Mechanonociceptors Respond to DAMGO after Local Pretreatment with Hypertonic Saline

To support the behavioral data, we studied the electrophysiological effects of hypertonic saline and opioids in in vitro skin nerve preparations (fig. 2).

A total of 81 C-fibers and 32 δ-fibers were studied in rats injected with either 0.9% (normal) or 10% (hypertonic) NaCl (saline). The proportion of polymodal C-fibers (heat sensitive), conduction velocities, and mechanical thresholds measured with von Frey hairs were not significantly different among the groups. Using a computer-controlled nanomotor, we applied mechanical stimuli (40–320 μm) to the receptive fields of each single C- and δ-fiber nociceptors. The mechanosensitivity of C- and δ-fibers from hypertonic saline-injected paws was not significantly different than that from normal saline-injected paws (figs. 2A and 3A). Application of DAMGO did not significantly alter the number of action potentials elicited by incremental mechanical stimuli in C- and δ-fiber nociceptors after 0.9% NaCl injections (fig. 2B and 3B). In contrast, DAMGO significantly reduced C-fiber nociceptor discharges in 10% NaCl-injected paws (fig. 2C). This effect was selective for C-fibers, as there was no change in mechanosensitivity in δ-fibers under the same conditions (fig. 3C). The effect was fully reversed by coapplication of equimolar concentrations of DAMGO and CTOP (fig. 2D).

Hypertonic Saline Decreases Immunoreactivity of Claudin-1

Tight junction protein composition was first studied in paw tissue from untreated animals (fig. 4). Among several tight junction proteins (claudin-1, occludin, claudin-5) that are expressed in the perineurium,9 we observed the most prominent changes in claudin-1. Claudin-1 immunoreactivity was observed in epidermis, perineurium, and blood vessels (figs. 4A-C). Occludin immunoreactivity was mostly seen in epidermis and blood vessels (figs. 4B, C). Claudin-5 immunoreactivity was only seen in blood vessels (fig. 4D). Claudin-1 distribution in blood vessels and perineurium was differentiated using staining for CGRP, a specific marker of sensory neurons. Peripheral nerves in a subcutaneous paw tissue expressing CGRP were surrounded by a layer of perineurial cells that were positive for claudin-1 immunoreactivity (fig. 4F). Blood vessels were only positive for claudin-1 immunoreactivity, but not for CGRP (fig. 4E). In the blood vessels, the intima of arterioles (fig. 4E), but less in venules (fig. 4C),

### Table 1. Baseline Mechanical Nociceptive Thresholds before Intraplantar Injection of NaCl and DAMGO

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>0.9</th>
<th>0.9</th>
<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
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<tbody>
<tr>
<td>DAMGO (μg)</td>
<td>—</td>
<td>30</td>
<td>—</td>
<td>10</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>PPT (g)</td>
<td>71.3 ± 1.4</td>
<td>73.3 ± 0.9</td>
<td>72.7 ± 1.9</td>
<td>67.7 ± 1.9</td>
<td>75.2 ± 0.8</td>
<td>73.8 ± 1.1</td>
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Data are presented as mean ± SEM. Two-way repeated measures ANOVA (n = 6/group); P > 0.05. DAMGO = [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin; PPT = paw pressure threshold.

![Fig. 2. Opioid agonist-induced inhibition of C-fiber mechanosensitivity following hypertonicity. (A–D) Rats were intraplantarly injected with 10% NaCl or 0.9% NaCl, and skin nerve preparations were established. (A) Mechanically evoked responses of C-fibers from 10% NaCl-injected (n = 27) rat skin were not different from 0.9% NaCl (n = 19) injected paw. (B–D) Responses of C-mechanonociceptors to a suprathreshold mechanical stimulus were recorded following exposure to synthetic interstitial fluid (SIF) (red squares) or [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) in SIF (10 μM, blue squares). (B) DAMGO had no effects on C-fiber responses when applied to receptive fields of 0.9% NaCl-injected paws (DAMGO, n = 18; SIF, n = 19). (C) DAMGO significantly reduced the mechanically evoked responses of C-fibers in 10% NaCl-injected paws (*P < 0.05; repeated-measures ANOVA, Bonferroni post hoc correction; DAMGO, n = 17; SIF, n = 27). (D) Coapplication of equimolar concentrations of DAMGO with a μ-opioid receptor-selective antagonist (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2,CTOP 10 μM) reversed the DAMGO effect (blue circle; SIF: red square, both n = 9; repeated-measures ANOVA, Bonferroni post hoc correction, P > 0.05). SIF = synthetic interstitial fluid.](http://pubs.asahq.org/anesthesiology/article-pdf/116/6/1323/257761/0000542-201206000-00029.pdf)
Facilitation of Peripheral Opioid Effects by Hypertonic Saline Is Dependent on Metalloproteinases

Intraplantar injection of the metalloproteinase inhibitor GM6001 either 10 min or 1 h (data not shown) before behavioral experiments completely prevented the enhancing effect of hypertonic saline on peripheral DAMGO-induced increase in mechanical nociceptive thresholds (fig. 6). GM6001 injection alone did not change mechanical nociceptive thresholds (PPT: baseline 67.71 ± 1.42 g; treated 74.58 ± 4.7 g, P > 0.05, n = 4, Student paired t test). Intraplantar treatment with 10% NaCl + GM6001 restored claudin-1 immunoreactivity compared with its reduction by hypertonic saline alone. Peripheral neurons were labeled with protein gene product 9.5 to confirm immunoreactivity of claudin-1 in peripheral neurons (fig. 7A). Semiquantification of the intensity of the claudin-1 staining by densitometric analysis of the sections confirmed a significant reduction after treatment with hypertonic saline, which was restored by pretreatment with GM6001 (fig. 7B). In claudin-1 Western blot, we confirmed that the antibody bound to a protein with the predicted size of claudin-1, 22 kDa. Hypertonic saline reduced claudin-1 content in the membrane fraction of subcutaneous paw tissue, which was restored with GM6001 pretreatment (figs. 7C, D). Western blot analysis by densitometry revealed a significant reduction (61.2%) after 10% NaCl treatment that was inhibited by GM6001 pretreatment.

Discussion

In this study we demonstrated that a single intraplantar injection of hypertonic saline facilitates the local antinociceptive effect of the hydrophilic MOR-opioid agonist DAMGO on mechanical nociceptive thresholds in noninflamed paw tissue for up to 6 h. The behavioral data are supported by electrophysiological recordings that showed an inhibition of mechanically evoked discharges in C-fibers by DAMGO following exposure to hypertonic saline. Furthermore, our data suggest that hypertonicity opens the perineurial barrier, facilitating access of hydrophilic opioids. Consistent with this notion, we observed a reduced immunoreactivity and protein content of the tight junction protein claudin-1 in the perineurium of peripheral nerves following injection of hypertonic solutions. This hypertonic effect was fully blocked by a metalloproteinase inhibitor in behavioral, immunohistochemical, and Western blot experiments.

In previous experimental and clinical studies, intraplantar injection of hydrophilic opioid agonists into noninflamed tissue did not induce antinociception. However, coinjection of hypertonic mannitol with opioids increased mechanical nociceptive thresholds. Here we demonstrate that intraplantar DAMGO, a hydrophilic peptide and MOR selective agonist, is only able to increase mechanical nociceptive thresholds if injected with or after another hypertonic solution, 10% saline. CTOP, a selective MOR antagonist, completely blocked the effect of DAMGO, suggesting that the analgesic effects are mediated by opioid receptor activa-
In the present experiments, the effect of hypertonic saline lasted for more than 6 h, whereas the concentration of saline in the tissue normalizes after 15 min, pointing toward structural changes of the perineurium to allow access of hydrophilic compounds. Since we used separate groups of rats to analyze the time course of the DAMGO effect, we excluded the possibility that the reduced response after perineurial DAMGO injection was because of reduced MOR function after repetitive activation. We show that the increase in barrier permeability occurs in small nerve bundles in the periphery. Opioid receptors are expressed along nerve trunks as well as in nerve terminals in peripheral tissue. Since some nerve terminals lack perineurium at their very tips (discussed in), our data argue that opioid receptors along the axon may play an important role for the generation of antinociceptive effects in noninflamed subcutaneous tissue. However, this has to be examined in future studies.

**Fig. 4.** Tight junction protein expression in the paw tissue. (A) Paw tissue from untreated rats was obtained and stained for claudin-1 (red) or claudin-5 (red) and occludin (green) as well as diamidinophenylindole (DAPI) for nuclei (blue) (magnification 10×). Different tissue types, such as epidermis (B), and peripheral nerves and blood vessels (C) known to express claudin-1 were identified by morphology based on DAPI nuclear staining within the structure (magnification 40×). (D) Claudin-5 immunoreactivity was observed in blood vessels, but not in the perineurium of the peripheral nerve (arrows) (magnification 40× and 100×, bar = 50 and 10 μm, respectively). (E, F) Paw tissue from normal rats was double-stained for claudin-1 (green) and calcitonin gene-related peptide (red) as well as DAPI for nuclei (blue) to identify sensory nerves in comparison with blood vessels. One representative example of blood vessels (E) and of peripheral nerves (F) is shown (magnification 100×, bar = 10 μm). (F) Negative control by omission of the primary antibody (anti claudin-1 antibody) is shown on the right (magnification 100×, bar = 10 μm). CGRP = calcitonin gene-related peptide; DAPI = diamidinophenylindole.
Several studies in humans and animals have shown that peripheral applications of low doses of opioids induce significant analgesia under inflammatory conditions. In previous electrophysiological studies, single unit activities of different C- and Aδ-fibers in normal rats were increased after local injection of glutamate into the receptive field, which was blocked by morphine and inhibited by naloxone. Peripheral inflammation by CFA sensitizes peripheral cutaneous nociceptors to mechanical and heat stimuli. In studies by Wenk et al., morphine directly inhibited mechanically and thermally activated C-fiber and C/Aδ-fiber nociceptors in inflamed skin 18 h after intraplantar injection of CFA.

Fig. 5. Decrease of claudin-1 staining in the perineurium of the paw following hypertonicity or inflammation. Rats were injected intraplantarly with 10% NaCl (center) or 150 μl complete Freund’s adjutant (right). Controls were from untreated rats (left). Subcutaneous tissue was stained for claudin-1 (red) and occludin (green). Nuclei were counterstained with diamidino-phenylindole (blue). Representative sections are shown (magnification 40×, bar = 20 μm). (B) Paw tissue was double-stained for claudin-1 (red) and protein gene product 9.5, a marker of peripheral neurons (green). Diamidino-phenylindole (blue) was used for counterstaining of nuclei. Omission of both primary antibodies as a negative control is included below. Representative sections are displayed (magnification 40×; bar = 20 μm). CFA = complete Freund’s adjuvant, DAPI = diamidino-phenylindole; PGP = protein gene product.
intramuscular and subcutaneous (plantar) tissue,37 and be-
injection in our experiments could be because of the short
cause we performed all our injections under anesthesia, in
humans.35,36 The lack of hyperalgesia after hypertonic saline
of hypertonic saline in behavioral or electrophysiological ex-
notwithstanding, our present results are in line with a selec-
tional effect was seen at higher sodium concentration.38 In
mediated G-protein activation in a manner inversely propor-
sodium inhibited spontaneous and agonist-occupied MOR-
besides, we did not observe claudin-5 in the perineurium as shown be-
the humans who express claudin-5 in the endoneurial
microvessels.42,43 Nerve damage by crush has been shown to
result in a loss of tight junction protein content in the
perineurium and opening of the perineurium.9 here, we
provide evidence that either inflammation induced by CFA
or hypertonic saline results in a loss of claudin-1 immuno-
activity. No change in occludin was observed. Since clau-
din-1 is a major sealing tight junction protein,10 it is conceiv-
able that its loss is crucial for the access of hydrophilic opioids
to the nociceptive neurons. In parallel to claudin-1 expres-
sion we demonstrated increased permeability after hyper-
tonic NaCl treatment, as shown before.6,5 the flux of HRP
involves both transcellular and paracellular routes but we did
not further examine this question because paracellular mark-
ers such as fluorescein-labeled dextran produced high back-
ground staining in our tissue. however, our approach might
be even superior as an indicator of permeability, as direct
functional effects were monitored. In inflammation interfer-
on-γ as well as tumor necrosis factor-α can cause a redistrib-
ution and internalization of tight junction proteins like claudin-1.44 Consequently, in CFA inflammation the lack of
immunoreactivity for claudin-1 could be because of internal-
ization of claudin-1 and/or its degradation.
several lines of evidence support the notion that hyper-
tonic solutions can affect tight junctions. In isolated frog
sciatic nerve, the permeability of 14C was enhanced after
exposure to hypertonic solution.7 At the blood-brain barrier,
osmotic challenges change the distribution of occludin,
ZO-1, and claudin-5.8 Hyperglycemia reduces ZO-1 and
occludin content in rat cerebrovascular cells. The latter find-
ing was accompanied by an increase in plasma MMP activity.
Metalloproteinases form a large group of enzymes secre-
ted as proenzymes.45 Substrates of MMP9 and MMP2
include most of the extracellular matrix components, which
are recognized by their secondary structure. several studies
demonstrated that MMPs effectively degrade tight junction
molecules such as occludin.11–13 In intestinal epithelial cells,
peritonic saline in vivo.5 our present study shows that the
effect of hypertonic saline on opioid-induced antinocicep-
tion can last up to 6 h. together, these data make a direct
influence on MOR function less likely and support the inter-
pretation that hypertonic saline may induce long-lasting
structural changes of the perineurial barrier.
the perineurium resembles the blood-brain barrier. An
epithelial origin is suggested because of the expression of
epithelial membrane antigen.6,9,40 the perineurium of
mixed peripheral nerves (including motor neurons) seems
to develop from central nervous system-derived glia.41
the perineurium surrounding the sciatic nerve expresses
claudin-1, claudin-5, occludin, VE-cadherin, and connexin43,5,9,40 We identified neurons in subcutaneous tissue by
either CGRP or protein gene product 9.5 and found them
surrounded by cells with claudin-1 immunoactivity. We
did not observe claudin-5 in the perineurium as shown be-
frastructure such as fluorescein-labeled dextran produced high back-
ground staining in our tissue. however, our approach might
be superior as an indicator of permeability, as direct
functional effects were monitored. In inflammation interfer-
on-γ as well as tumor necrosis factor-α can cause a redistrib-
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ization of claudin-1 and/or its degradation.
several lines of evidence support the notion that hyper-
tonic solutions can affect tight junctions. In isolated frog
sciatic nerve, the permeability of 14C was enhanced after
exposure to hypertonic solution.7 At the blood-brain barrier,
osmotic challenges change the distribution of occludin,
ZO-1, and claudin-5.8 Hyperglycemia reduces ZO-1 and
occludin content in rat cerebrovascular cells. The latter find-
ing was accompanied by an increase in plasma MMP activity.
Metalloproteinases form a large group of enzymes secre-
ted as proenzymes.45 Substrates of MMP9 and MMP2
include most of the extracellular matrix components, which
are recognized by their secondary structure. several studies
demonstrated that MMPs effectively degrade tight junction
molecules such as occludin.11–13 In intestinal epithelial cells,
interferon-γ induces MMP-mediated degradation of claudin-2. However, claudin-1 proteolysis has not been examined. In vivo changes in the blood-brain barrier that function under pathologic conditions are in part mediated by MMPs. For example, in focal ischemia in the rat, MMP activity is increased, and treatment with an MMP inhibitor reversed the degradation of claudin-5 and occludin. In addition, it has been shown that hypertonic solutions can increase the activity of MMPs in corneal epithelial cells. Consistently, we show here that a broad-spectrum MMP inhibitor reversed the effect of hypertonic saline on the accessibility of MOR and on the immunoreactivity of claudin-1 in the perineurium. The particular MMPs involved, as well the exact mechanism of action, needs to be explored in future studies.

In summary, we have shown that hypertonicity facilitates exogenous peripheral opioid actions in noninflamed tissue both in behavioral and electrophysiological experiments. Hypertonic saline also decreases the immunoreactivity of claudin-1 in the perineurium. These effects can be fully blocked by an MMP inhibitor. Specifically and reversibly modulating tight junction proteins in the perineurium might also allow controlled access of other substances for treatment.

Fig. 7. Metalloproteinase inhibition blocks hypertonicity-induced decrease of claudin-1 expression. Rat paws were treated with 100 μl hypertonic saline, or with hypertonic saline together with a metalloproteinase inhibitor (0.2 mg GM6001). Tissue was stained for claudin-1 (red) and protein gene product 9.5 (green) (A). Nuclei were counterstained with diamidinophenylindole (DAPI, blue). Representative sections are shown (magnification 40×; bar = 10 μm). (B) The relative intensity of the staining was semiquantified by densitometric analysis of the stained sections (n = 12, *P < 0.05, ANOVA, post hoc Dunnet’s method vs. control). (C) Tissue was analyzed by Western blot. Representative blots are shown. (D) Western blot analysis by densitometry (integrated density value, relative to β-actin) revealed a significant reduction after 10% NaCl treatment that was inhibited by GM6001 pretreatment (n = 4 or 5/group, *P < 0.05, ANOVA, post hoc Dunnet’s method vs. control). IDV = integrated density value; PGP = protein gene product.
of pain or other diseases. However, studies on long-term safety and toxicity will be needed before clinical application.

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