Transient opening of the perineurial barrier for analgesic drug delivery

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Selective targeting of sensory or nociceptive neurons in peripheral nerves remains a clinically desirable goal. Delivery of promising analgesic drugs is often impeded by the perineurium, which functions as a diffusion barrier attributable to tight junctions. We used perineurial injection of hypertonic saline as a tool to open the perineurial barrier transiently in rats and elucidated the molecular action principle in mechanistic detail: Hypertonic saline acts via metalloproteinase 9 (MMP9). The noncatalytic hemopexin domain of MMP9 binds to the low-density lipoprotein receptor-related protein-1, triggers phosphorylation of extracellular signal-regulated kinase 1/2, and induces down-regulation of the barrier-forming tight junction protein claudin-1. Perisciatic injection of any component of this pathway, including MMP9 hemopexin domain or claudin-1 siRNA, enables an opioid peptide ([D-Ala2,*N*-Me-Phe4, Gly5-ol]-enkephalin) and a selective sodium channel (NaV1.7)-blocking toxin (ProToxin-II) to exert antinociceptive effects without motor impairment. The latter, as well as the classic TTX, blocked compound action potentials in isolated nerves only after disruption of the perineurial barrier, which, in return, allowed endoneurally released calcitonin gene-related peptide to pass through the nerve sheaths. Our data establish the function and regulation of claudin-1 in the perineurium as the major sealing component, which could be modulated to facilitate drug delivery or, potentially, reseal the barrier under pathological conditions.

regional anesthesia | blood-nerve barrier | C-fiber | two path impedance spectroscopy

Selective blockade of sensory, particularly nociceptive, signaling remains an important yet clinically unattainable goal for pain treatment. Local anesthetics unselectively block sodium channels, leading to impaired action potential propagation in nociceptive, sensory, and motor neurons, which results in disturbed sensory and motor control of the limb. So far, coapplication of the impermeable lidocaine derivative QX-314 with the transient receptor potential channel vanilloid 1 (TRPV1) agonist capsaicin (1) or with nonselective surfactants has been suggested to induce prolonged nociceptive- and sensory-selective nerve blockade (2) without motor impairment. Although promising, these approaches induce pain on injection, lack full selectivity, or may confer neurotoxicity.

Voltage-gated sodium channels NaV 1.7, 1.8, and 1.9 (3, 4) play important roles in nociceptive nerve conduction, but specific antagonists, such as the tarantula venom peptide Protoxin-II (ProTx-II; Peptides International), are not effective in vivo because of lack of permeability of the perineurial barrier (5). Opioid receptors are also selectively expressed on nociceptive neurons at the nerve terminal (6, 7), at pre- and postsynaptic sites of nociceptive neurons in the dorsal horn, and in the axon (8). The synthetic peptide [D-Ala2,*N*-Me-Phe4,Gly5-ol]-enkephalin (DAMGO) is a specific agonist for μ-opioid receptors (MORs), whereas [D-Pen2, d-Pen5]-enkephalin (DPDPE) binds to δ-opioid receptors (DORs). DAMGO and DPDPE, as well as the hydrophilic opioid morphine (a MOR agonist), are effective analgesics when injected locally into inflamed tissue (9), but perineurial injection does not change thermal nociceptive thresholds in rats (10).

Peripheral nerves are surrounded by the perineurium, which is composed of a basal membrane with a layer of perineurial cells and tight junctions limiting paracellular permeability (11). The tight junction proteins claudin-1, claudin-5, and occludin are expressed in the perineurium and associated with permeability changes of the perineurium in a model of crush injury of the nerve (12, 13). Hypertonic solutions have been used to open barriers to enhance drug delivery to the brain (14). They also increase perineurial permeability and facilitate peripheral opioid antinociception at nerve terminals (15, 16). Metalloproteinases (MMPs) modulate the permeability of the blood–brain barrier (17). Some of the MMPs carry a hemopexin domain (PEX), which is responsible for proper substrate recognition and activation of the enzyme, as well as its final internalization and degradation. MMP9 PEX also binds to the scavenger receptor lipoprotein receptor-related protein 1 (LRP-1). LRP-1 attaches to multiple targets, such as lipoproteins, ECM glycoproteins, and protease/inhibitor complexes. MMP9 PEX binding to LRP-1 induces Schwann cell migration [e.g., in nerve injury via extracellular signal-regulated kinase 1/2 (ERK1/2) activation] (18).

In this study, we examined whether opening of the perineurial barrier can be enforced to facilitate drug delivery to the peripheral nerve. Our findings revealed unique molecular targets for the specific and controlled opening of the perineurium (e.g., to allow for selective antinociception).

Results

Hypertonic Saline Functions as a Permeation Enhancer to Enable Blockade via Sensory- and Nociception-Specific Drugs. Wistar rats received a perisciatic injection of sodium channel blockers together with hypertonic [10% (vol/vol)] or isotonic (0.9%) saline.

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TTX and the NaV 1.7-specific blocker ProTx-II resulted in a dose-dependent rise of the paw withdrawal threshold to noxious mechanical pressure (Randall–Sellito test; Ugo Basile) when coinjected with hypertonic saline (Fig. 1 *A*–*C*) but not with isotonic saline (Fig. 1*A*). No effects were observed contralaterally or with hypertonic saline alone (Fig. 1*A*). To support the in vivo data, compound action potentials were examined in isolated sciatic nerves. TTX or ProTx-II did not block compound action potentials in the untreated intact nerve except for a small reduction probably attributable to minor leaks caused by avulsion of small nerve branches. Hypertonic synthetic interstitial fluid (10× NaCl-SIF) treatment for 5–10 min resulted in a transient strong reduction or abolishment of the A- and C-volleys, which recovered to a variable extent during the subsequent 10 min of washout. After hypertonic but not isotonic treatment, a substantial reduction of the area under the curve of the C- and A-volleys in sciatic nerves was regularly achieved with superfusion of TTX or ProTx-II, whereby the ProTx-II blockade of the A-fibers was less complete than that of the C-fibers and the TTX blockade of both fiber types (Fig. 1 *D* and *E*).

Similarly, perisciatic injection of the hydrophilic MOR agonist DAMGO or the DOR-specific agonist DPDPE did not change mechanical nociceptive thresholds unless hypertonic saline was injected concomitantly (Fig. 2 *A*–*C*). Both effects were specifically and dose-dependently blocked by the respective opioid receptor antagonists naloxone (Fig. 2*D*) and naltrindole (Fig. 2*E*).

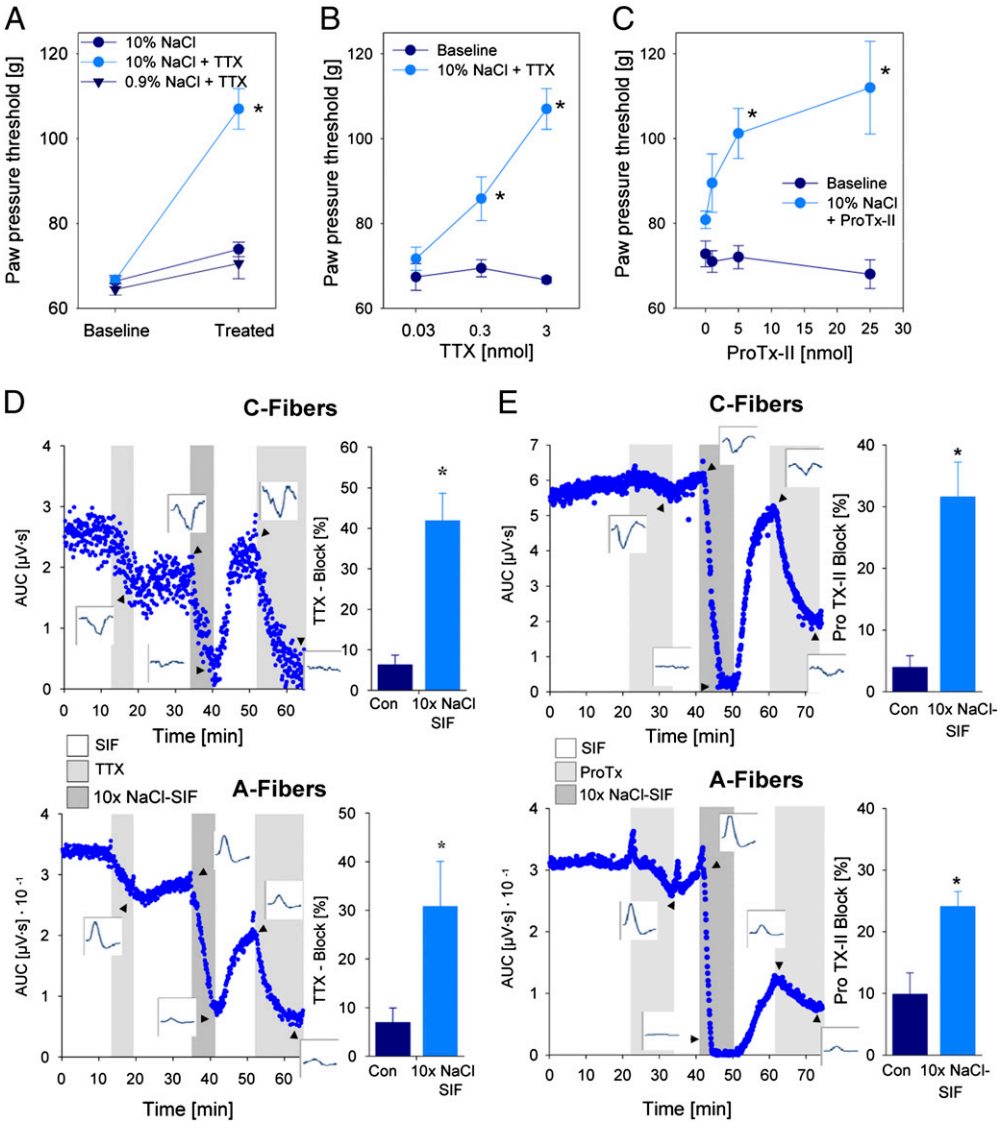

Fig. 1. Perisciatic coapplication of hypertonic saline solution with TTX- or ProTx-II increases mechanical nociceptive thresholds and blocks compound action potentials. (*A*–*C*) Measurement of paw pressure thresholds in rats before (baseline) and 10 min after perisciatic injection of 0.9% or 10% NaCl together with TTX (*B* and *C*) or ProTx-II (*C*) (*$P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 6$), revealing an antinociceptive effect of TTX and ProTX-II only after 10% NaCl but not after 0.9% NaCl. (*D* and *E*) Recordings of compound action potentials from isolated rat sciatic nerves were obtained, and the area under the curve (AUC) of C-fibers (*Upper*) and A-fibers (*Lower*) of representative samples, including inserts with original compound action potential recordings, are shown. Nerves were first treated with 0.5 µM TTX (*D*; $n = 7$, light gray background) or 5 µM ProTx-II (*E*; $n = 6$, light gray background) for 10 min and then incubated with 10× NaCl-SIF (dark gray background) for 10 min and subsequently exposed again to the same concentration of TTX or ProTx-II (light gray background). Between these treatments, nerves were allowed to recover in SIF (white background). The percentage of block was significantly enhanced 10 min after addition of TTX or ProTx-II only after 10× NaCl-SIF or 10× SIF pretreatment (*$P < 0.05$, Wilcoxon matched pairs test). All data are mean ± SEM. Con, control.

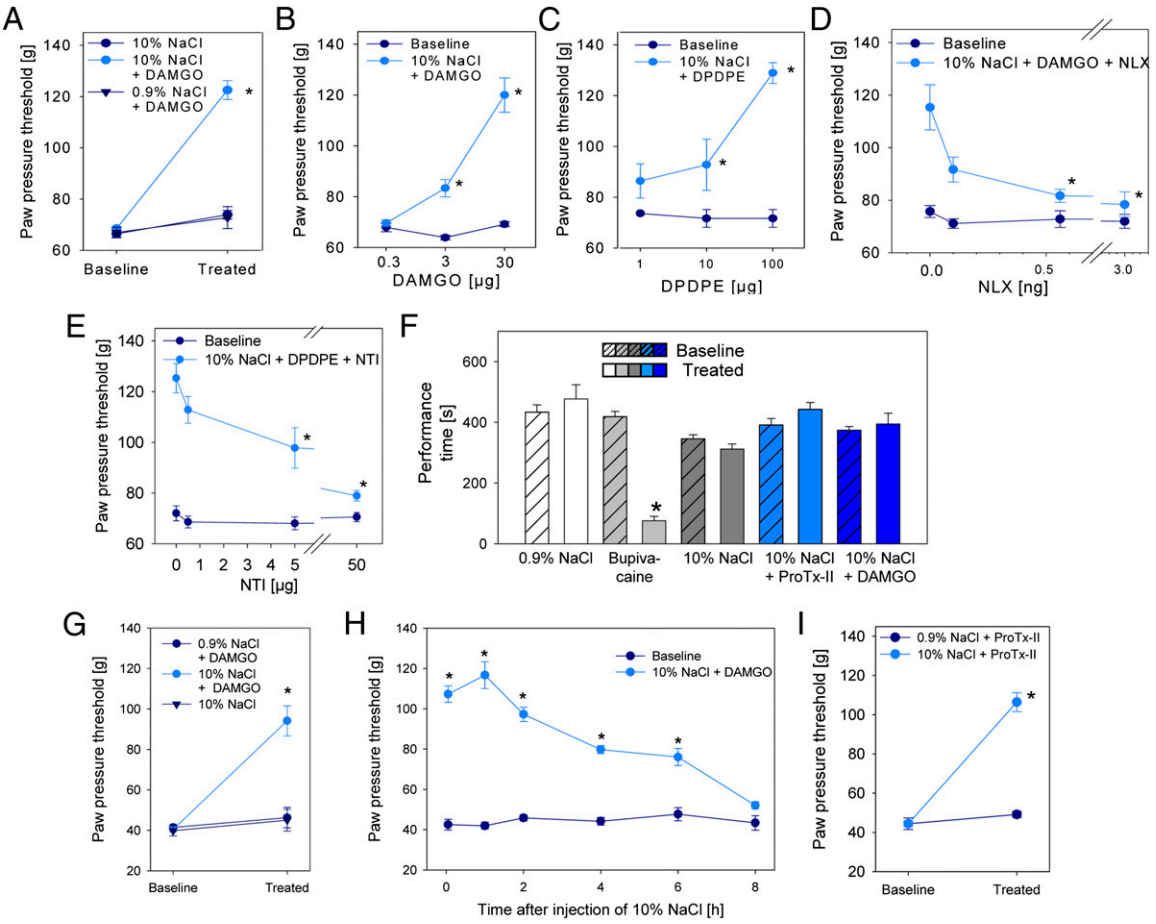

Fig. 2. Perisciatic injection of hypertonic saline with MOR or DOR agonist augments mechanical nociceptive thresholds in naive rats, as well as in rats with CFA inflammation. Rats were injected with 0.9% or 10% NaCl with or without 30 µg of DAMGO (MOR agonist) (*A* and *B*) or 100 µg of DPDPE (DOR agonist) (*C*). Mechanical nociceptive thresholds (paw pressure thresholds) increased 10 min only after coinjection with hypertonic saline. No change in paw pressure threshold was seen in contralateral paws. To test for opioid receptor specificity, 10% NaCl was injected with the MOR and DOR agonists with or without their respective opioid receptor antagonist MOR [naloxone (NLX)] (*D*) or DOR [naltrindole (NTI)] (*E*) (all $*P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 6$). (*F*) Motor function was tested using a rotarod before and after treatment with 10% NaCl with or without DAMGO or ProTx-II. Bupivacaine was used as positive control, and 0.9% NaCl as was used as a negative control ($*P < 0.05$, two-way ANOVA; $n = 4–6$). All data are mean ± SEM. (*G* and *I*) In rats with 4 d of CFA inflammation, paw pressure thresholds were quantified before and 10 min after perisciatic injection of 0.9% or 10% NaCl with or without 30 µg of DAMGO or 5 µM ProTx-II. (*H*) After an initial injection of 10% NaCl next to the sciatic nerve, mechanical nociceptive thresholds were repeatedly measured over a time course of 8 h. Paw pressure thresholds were obtained 10 min after injection of 30 µg of DAMGO at the indicated time points ($*P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 6$; independent groups at all time point). All data are mean ± SEM.

None of the treatment strategies resulted in significant impairment of the motor function using rotarod testing (Fig. 2*F*); the local anesthetic bupivacaine was used as a positive control. Coinjection of DAMGO or ProTx-II together with hypertonic saline not only elicited antinociception in naive rats but in those with hind paw inflammation induced by complete Freund's adjuvant (CFA) (Fig. 2 *G* and *I*).

Increased Permeability and Decreased Tight Junction Protein Expression in the Perineurium After Perisciatic Injection of Hypertonic Saline. To explore the duration of the permeabilizing effect of hypertonic saline, DAMGO was injected at different time points after perisciatic injection of hypertonic saline in independent groups of rats. A significant increase of the mechanical nociceptive thresholds was observed for up to 4 h in naive rats (Fig. 3*A*) and for up to 6 h in rats with CFA hind paw inflammation (Fig. 2 *H*). Next, we evaluated the permeability of the barrier. The isolated intact sciatic nerve of the rat releases minor amounts of calcitonin gene-related peptide (CGRP) under baseline conditions and on stimulation with capsaicin (Fig. 3*B*), which originates from epineurial nerve endings (nervi nervorum) of the nerve sheath, although the lipophilic TRPV1 agonist passes the nerve sheath easily and stimulates axons of passage (19). The desheathed nerve releases massive amounts of CGRP from the axons of primary afferent C-fibers on chemical (capsaicin) stimulation, and this response is not altered by previous hypertonic treatment (positive control). Treatment of intact rat nerves with 10% NaCl-SIF enabled CGRP permeation through the nerve sheath under basal conditions, as well as on stimulation with capsaicin (Fig. 3*B*). In contrast, the perineurial barrier of mice is rather permeable, because on electrostimulation of sciatic nerves with intact nerve sheath, only 62% of the axonally released CGRP was retained but 38% was allowed to pass through into the incubation fluid (*SI Text*). When untreated sciatic nerves of rats were immersed in Evan's blue albumin (EBA), red fluorescent staining was confined to the perineurium, whereas after in vivo treatment with 10% NaCl, EBA was also widely distributed throughout the endoneurium inside the nerve (Fig. 3*C*). To study the function of tight junction proteins, we chose to examine claudin-1, claudin-5, and occludin expression. Immunohistochemical staining demonstrated that only claudin-1 labeling in the

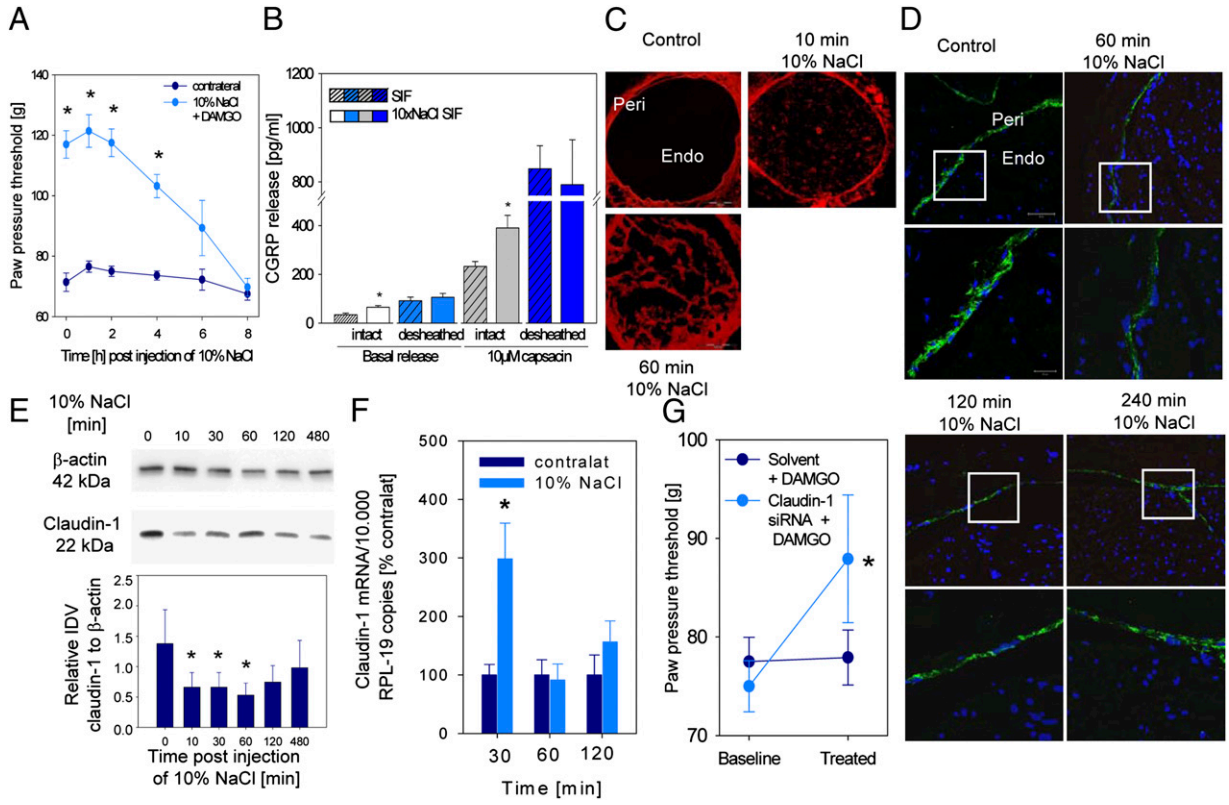

Fig. 3. Enhancing effects of hypertonic saline depend on altered claudin-1 expression in the sciatic nerve. (*A*) Different groups of rats were perisciatically injected with 10% NaCl at the same time point, and, subsequently, a single group received DAMGO (30 μg perisciatically) at one of the indicated time points. Paw pressure thresholds were increased up to 6 h after opioid injection (*$P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 6$). (*B*) Measurement of CGRP in the supernatant after incubation of desheathed isolated sciatic nerves with 10× NaCl-SIF pretreatment before and after stimulation with capsaicin is shown in comparison to intact sciatic nerves (*$P < 0.05$, one-way ANOVA; $n = 4$). (*C*) Penetration of EBA into the sciatic nerve increased 10 and 60 min after application of 10% NaCl compared with control (representative example) Endo, endoneurium; Peri, perineurium. (Scale bar: 250 nm.) (*D*) (*Upper*) Immunoreactivity for claudin-1 (green) and staining of the nucleus (DAPI, blue) were performed in the perineurium under control conditions, as well as 60–240 min after injection of 10% NaCl (representative samples). (Scale bar: 50 μm.) (*Lower*) Higher magnifications from the original section are shown. (Scale bar: 20 μm.) (*E*) Claudin-1 protein expression in the membrane fraction of the sciatic nerve was reduced at early time points after perisciatic injection of 10% NaCl. Expression was quantified by densitometry in proportion to β-actin as a loading control for the relative integrated density value (IDV) (*$P < 0.05$, ANOVA; $n = 4$). (*F*) Claudin-1 mRNA expression was measured by semiquantitative PCR after 10% NaCl injection [*$P < 0.05$ vs. contralateral (contralat) side, two-way repeated ANOVA; $n = 9–13$]. (*G*) Rats were treated with siRNA against claudin-1 for 2 d. Paw pressure thresholds were increased when measured 10 min after an additional injection of DAMGO (*$P < 0.05$, ANOVA, Student–Newman–Keuls method; $n = 7$). All data are mean ± SEM.

perineurium was reduced within 1 h after perineurial injection of hypertonic saline and remained low up to 4 h (Fig. 3*D*). No internalization of claudin-1 immunocomplexes was observed. A significant reduction of claudin-1 protein content within 10 min after application of 10% NaCl was confirmed in the membrane fraction of sciatic nerves (Fig. 3*E*). No claudin-1 fragments were seen. On the transcriptional level, claudin-1 mRNA expression in the sciatic nerve was significantly up-regulated within 30 min after hypertonic saline injection (Fig. 3*F*). No change was seen in occludin and claudin-5 mRNA (Fig. S1). In addition, repeated perisciatic application of siRNA against claudin-1 modestly but significantly enhanced the efficacy of the MOR agonist DAMGO (Fig. 3*G*). siRNA treatment reduced claudin-1 immunoreactivity in the sciatic nerve (Fig. S1). To examine the impact of treatment with hypertonic solutions on nerve integrity, we examined the histology of the nerve, as well as nociceptive thresholds, by means of infiltration with CD68 macrophages over a period of 1 wk. No signs of histological nerve damage or enhanced nociceptive function were observed (Fig. S2).

MMP9 PEX Domain Functions as a Key Intermediate Step Regulating the Opening of the Perineurial Barrier. To explore the mechanism of hypertonic saline-induced opening of the perineurium, a broad-spectrum MMP inhibitor (GM6001) and an MMP9-specific inhibitor were injected at the sciatic nerve together with hypertonic saline. Both inhibitors dose-dependently blocked the antinociceptive effects of DAMGO coinjected with hypertonic saline (Fig. 4 *A* and *C*), whereas an MMP3-specific inhibitor was ineffective (Fig. S3*A*), although MMP9, MMP3, and MMP7 all were expressed in the perineurium (Fig. 4*B* and Fig. S3 *C* and *E*). Claudin-1 protein content in the membrane fraction of the sciatic nerve and claudin-1 mRNA expression in the nerve were unchanged if the MMP9 inhibitor was coinjected perisciatically with hypertonic saline (Fig. 4 *D* and *E*). In the absence of hypertonic saline, perisciatic coinjection of recombinant MMP9, but not MMP3 or MMP7 facilitated DAMGO-induced elevations in mechanical nociceptive thresholds (Fig. 4*F* and Fig. S3 *B* and *D*). Recombinant MMP9 injected at the sciatic nerve also significantly decreased claudin-1 protein content in the nerve and up-regulated claudin-1 mRNA expression (Fig. 4 *G* and *H*). No change was seen in occludin or claudin-5 expression (Fig. S1 *B* and *C*).

We next postulated that MMP9 modulates claudin-1 through signaling via the PEX domain because no claudin-1 degradation products were seen in Western blots (Fig. 3*D*). Coinjection of hypertonic saline with anti-MMP9 PEX antibody dose-de-

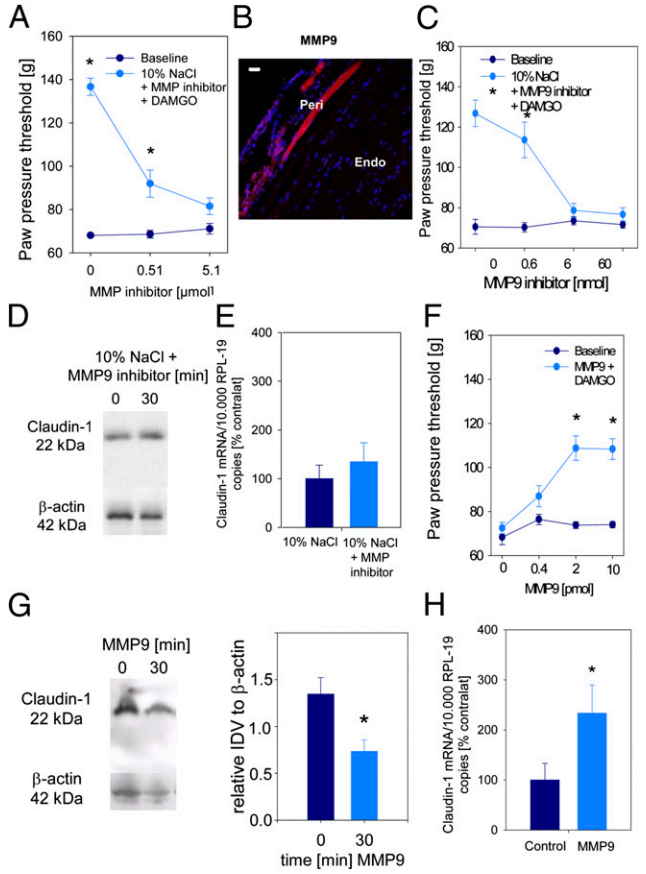

Fig. 4. MMP9 enhances the antinociceptive effects of perineurial opioids and reduces the expression of claudin-1 in the sciatic nerve. Mechanical nociceptive thresholds were quantified before (baseline) and 10 min after injection of DAMGO with 10% NaCl together with the broad-spectrum MMP inhibitor GM6001 (*A*) or a specific MMP9 inhibitor (*C*) or after injection of recombinant MMP9 alone (*F*) (*$P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 6$). (*B*) Immunoreactivity of MMP9 (red) was observed in the perineurium of the sciatic nerve. DAPI staining is shown in blue. Endo, endoneurium; Peri, perineurium. (Scale bar: 40 µm.) Claudin-1 content in the membran fraction of sciatic nerves remained unchanged 30 min after perineurial injection of hypertonic saline with an MMP9 inhibitor (6 nmol) (*D*), whereas it decreased after injection of 2 pmol of recombinant MMP9 in the absence of hypertonic saline [quantified in relative integrated density value (IDV); *$P < 0.05$ vs. control, ANOVA, Student–Newman–Keuls method; $n = 4$] (*G*). mRNA expression of claudin-1 was measured in the sciatic nerve and remained the same after perisciatic injection of 10% NaCl and a 10-min pretreatment with MMP inhibitor (*E*), whereas it increased after injection of 2 pmol of recombinant MMP9 (*H*) [normalized data % contralateral (contralat); *$P < 0.05$ vs. contralateral side, Mann–Whitney rank sum test; $n = 7$ or 14]. All data are mean ± SEM.

pendently reduced hypertonic saline with DAMGO-induced increases in mechanical nociceptive threshold (Fig. 5*A*). Purified recombinant MMP9 PEX contains the PEX domain but is devoid of the MMP9 catalytic domain (20). It dose-dependently facilitated DAMGO and ProTx-II induced increases in mechanical nociceptive thresholds (Fig. 5 *B* and *C*). Anti-MMP9 PEX antibodies blocked the effects of recombinant MMP9 PEX (Fig. 5*D*). Within the first hour, claudin-1 content significantly decreased after perisciatic MMP9 PEX application (Fig. 5*E*). MMP9 PEX in vivo pretreatment facilitated a block of C-fibers and A-fibers by TTX in ex vivo compound action potential recordings using TTX (Fig. 5*F*). No change in compound action potentials was observed in control nerves treated with TTX.

MMP9 PEX Domain Targets LPR-1 Expressed in the Perineurium. MMP9 PEX binds to LRP-1 on, for example, Schwann cells (18). Immunohistochemical staining showed colocalization of LRP-1 with claudin-1 in the perineurium (Fig. 6*A*). LRP-1 mRNA was expressed in the sciatic nerve (Fig. 6*B*). LRP-1 can be blocked with the chaperone receptor-associated protein (RAP) (18). Perisciatic application of hypertonic saline with RAP or MMP9 PEX with RAP completely blocked the effect of the hypertonic saline or MMP9 PEX on DAMGO-induced increases in mechanical nociceptive threshold (Fig. 6 *C* and *D*). Because no perineurial cell line with intact barrier properties is available, we used the human colonic epithelial cell line HT-29/B6 because its transepithelial resistance is dependent on claudin-1 to explore MMP9 PEX function in vitro. The putative receptor for MMP9 PEX, LRP-1, was expressed in HT-29/B6 cells on the transcriptional level (Fig. 6*E*). Incubation of HT-29/B6 with MMP9 PEX for 1 h significantly decreased the paracellular resistance, whereas the transcellular and resulting epithelial resistances remained unchanged (Fig. 6*F*). This drop in resistance was paralleled by an increase in the permeability for the paracellular marker fluorescein (330 Da) (Fig. 6*G*). Treatment of HT-29/B6 cells with MMP9 PEX decreased immunoreactivity of claudin-1 but not of occludin, as seen by immunohistochemistry (Fig. 6*H*). Together, these data suggest that the PEX domain of MMP9 can open perineurial barriers, as well as epithelial barriers, via interaction with LRP-1.

In addition to its endocytotic function, LRP-1 activates several signaling molecules, including kinases like ERK1/2 as seen in Schwann cells in neuropathic pain (18). Perisciatic injection of hypertonic saline transiently enhanced ERK phosphorylation in the sciatic nerve beginning 5 min after application (Fig. 7*A*). ERK protein expression remained unchanged. The increase of ERK phosphorylation was confined to the perineurium (Fig. 7*B*). Application of recombinant MMP9 perisciatically also resulted in phosphorylation of ERK within the first 30 min (Fig. 7*C*). Perisciatic injection of an ERK inhibitor, PD98059, dose-dependently and almost completely blocked the ability of hypertonic saline to facilitate DAMGO-induced increases in mechanical nociceptive thresholds (Fig. 7*D*) and blocked the reduction in claudin-1 content (Fig. 7*E*) after hypertonic saline injection at the sciatic nerve. In contrast, neither perineurial treatment with hypertonic saline solution nor MMP9 changed the phosphorylation of the Akt kinase in the perineurium (Fig. S3).

Discussion

The perineurial barrier impedes the access of various hydrophilic drugs that could lead to selective blockade of sensory or even nociceptive fibers. Here, we explored the molecular mechanisms underlying the regulated and reversible opening of the perineurial barrier of the peripheral nervous system for drug delivery. Coinjection of hypertonic saline solution as a tool together with a selective blocker of the voltage-gated sodium channel NaV 1.7 (ProTx-II) induced antinociception in behavioral experiments and blockade of nerve conduction in ex vivo neurophysiological studies. Similarly, opioid receptor agonists (DAMGO or DPDPE) induced a specific antinociceptive effect in behavioral pain experiments. MMP9 was a key regulatory step, and its hemopexin domain bound to LRP-1 on perineurial cells. LRP-1 activation induced ERK1/2 signaling in the perineurium and led to a reduced content of the tight junction protein claudin-1 sealing the paracellular cleft of the perineurium. Taken together, we have described a unique approach for drug delivery to peripheral nerves and, potentially, for targeted pain control in patients.

Sensory- and nociception-specific blockade was studied using sodium channel blockers, as well as opioids. TTX blocks sodium channels on nociceptors, as well as on nonnociceptors (21), whereas ProTx-II is more specific (5). Both sodium channel blockers were successfully used in vitro to block propagation of

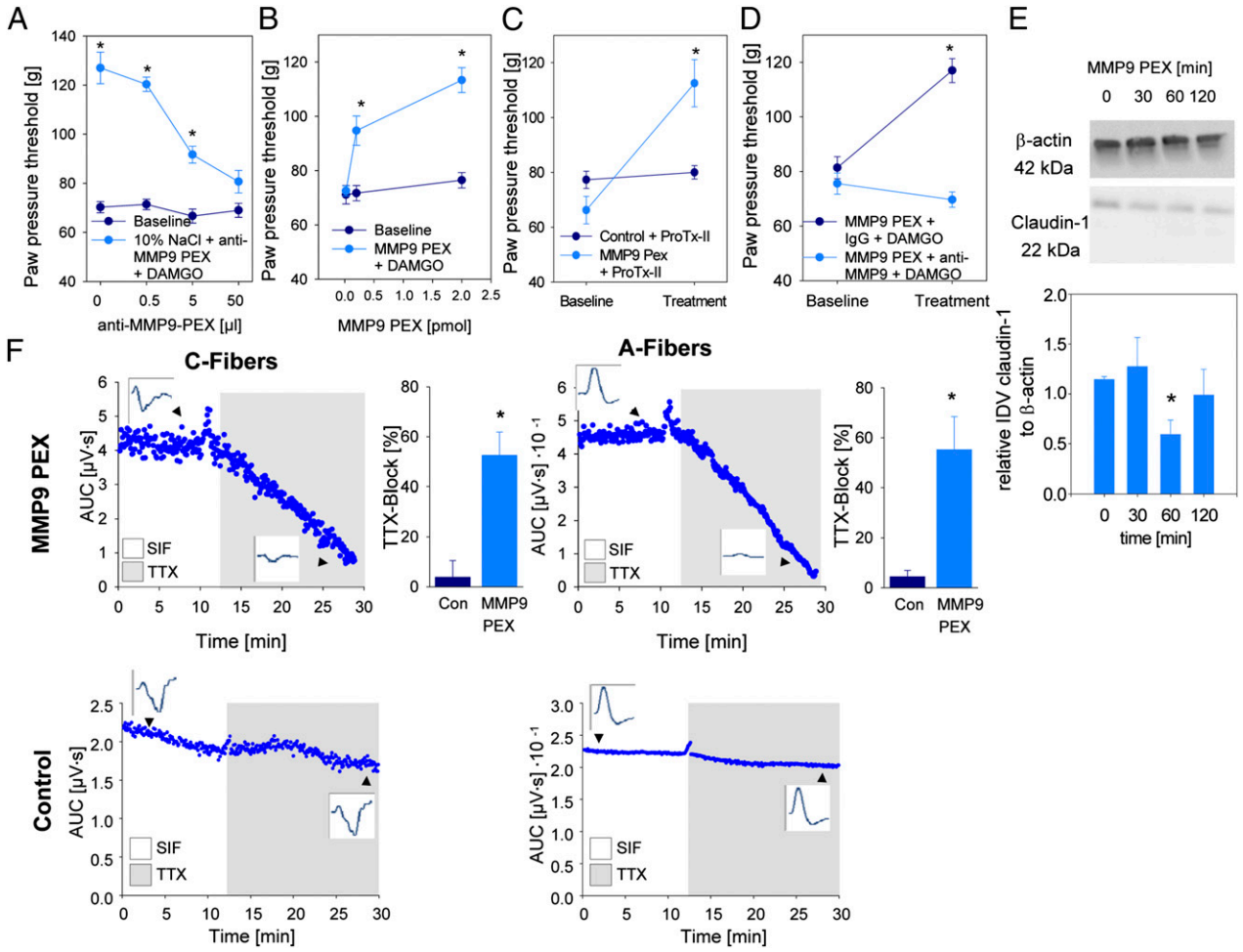

Fig. 5. MMP9 PEX faciliates opioid-induced antinociception and inhibition of nerve conductivity at the sciatic nerve. Rats received a perisciatic injection of an anti-MMP9 PEX antibody followed by DAMGO in 10% NaCl (*A*) or recombinant MMP9 PEX (2 pmol) followed by DAMGO (30 µg) (*B*) or ProTx-II (*C*). Mechanical nociceptive thresholds were determined 10 min later. (*D*) Specificity of MMP9 PEX was evaluated by perisciatic injection of DAMGO with 2 pmol of MMP9 PEX pretreated with 50 µL of anti-MMP9 PEX antibodies (control: 50 µL of control IgG; $*P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 6$). (*E*) Claudin-1 protein content in the membrane fraction of the sciatic nerve decreased 30–120 min after perineurial injection of 2 pmol of MMP9 PEX [relative integrated density value (IDV) of claudin-1 vs. β-actin; $*P < 0.05$ vs. baseline, ANOVA, Student–Newman–Keuls method; $n = 4$]. (*F*) Representative recordings of ex vivo compound action potentials are shown from rat sciatic nerve after in vivo pretreatment with 2 pmol of MMP9 PEX compared with control (Con) untreated sciatic nerves. The area under the curve (AUC) of C-fiber and A-fiber compound action potentials, as well as inserts with original compound action potential recordings, are shown before and during treatment with 0.5 µM TTX (gray background). This pretreatment resulted in a significant percentage of blockade 20 min after addition of TTX ($*P < 0.05$ Wilcoxon matched pairs test; $n = 4$). All data are mean ± SEM.

compound action potentials in A-fibers and C-fibers. Future possible candidates are spider toxins or conotoxins (22). Opioid receptors have been demonstrated in nerve axons and are specifically expressed on nerve endings (8, 23). They are functional under pathophysiological conditions like nerve injury, because endogenous opioid peptides produced by infiltrating immune cells elicit opioid receptor-selective antinociception (8). Under normal conditions in the rat model, as well as in human patients, the function of opioid receptors along the axon remains controversial (10, 24). We propose that the apparent lack of efficacy might be attributable to the impermeability of the perineurial barrier to allow sufficient access of the injected opioid to nociceptors. Perineurial opioids and ProTx-II were not only ineffective in naive rats but in rats with CFA hind paw inflammation, whereas coapplication with hypertonic saline allowed for antinociceptive effects under both conditions. Peripheral painful inflammation increases solute permeability through the blood–brain barrier and changes the protein composition of the tight junction, including a decrease in occludin, whereas claudin-3 and claudin-5 are increased and claudin-1 remains unchanged (25, 26). However, the locally restricted hind paw inflammation in our experiments did not functionally break the perineurial barrier further proximally. Such a break may well occur on neuritis or perineural inflammation, which are subjects of future studies. In the clinical perioperative settings for regional anesthesia, most patients do not usually experience profound peripheral inflammation preoperatively and are often pain-free, more comparable to naive rats than to rats with CFA inflammation.

Hypertonic saline has previously been used to open barriers like the blood–brain barrier (27) or the perineurium in peripheral nerves (15, 16). Using in vitro neuronal CGPR release, as well as EBA immersion, we confirmed that the barrier was opened after treatment with hypertonic saline. The lack of hyperalgesia after hypertonic saline injection in our experiments could be attributable to the short duration of the effect, the anatomical difference between intramuscular and subcutaneous (perineurial) tissue, and the fact that we performed all our injections under anesthesia in contrast to others. Although the hypertonic treatment in vitro blocked nerve conduction completely but transiently, the injection of 10% NaCl in vivo did not

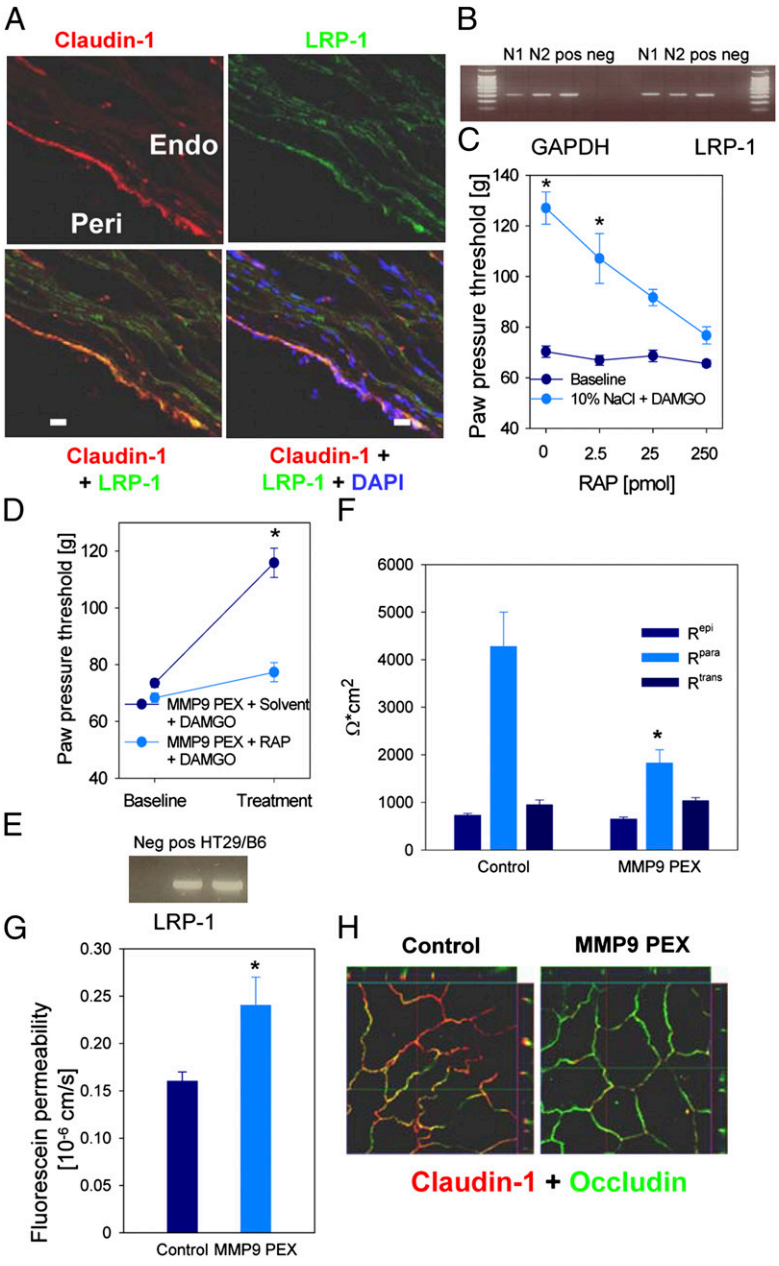

Fig. 6. MMP9 PEX domain interacts with low-density LRP-1, induces barrier permeability, and mediates the antinociceptive effects of hypertonic solution and DAMGO. (*A*) Representative examples of immunohistochemical staining of the untreated sciatic nerve for claudin-1 (*Upper Left*, red), LRP-1 (*Upper Right*, green), and coexpression of claudin-1 with LRP-1 (*Lower Left*, orange) in the perineurium. (*Lower Right*) Nuclei were stained with DAPI (blue). Endo, endoneurium; Peri, perineurium. (Scale bar: 40 μm.) (*B*) mRNA expression of LRP-1 in two different sciatic nerves [N1 and N2; positive (pos) control: liver; negative (neg) control]. The increases of paw pressure thresholds were blocked when rats were treated with the LRP antagonist RAP and DAMGO with 10% NaCl (*C*) or with 2 pmol of MMP9 PEX (*D*) before and 10 min after perisciatic injection (*$P < 0.05$, ANOVA, Student–Newman–Keuls method; $n = 6$). (*E*) Effect of MMP9 PEX was examined on a claudin-1–dependent colon carcinoma cell line HT-29/B6, which also expressed the LPR-1 mRNA. (*F*) HT-29/B6 cells treated with 150 pmol of MMP9 PEX epithelial (R^{epi}), paracellular (R^{para}), and transcellular resistances (R^{trans}) were quantified by two-path impedance spectroscopy. Only R^{para} decreased after MMP9 PEX treatment. (*G*) Permeability for the paracellular marker fluorescein (332 Da) in HT-29/B6 cells was increased after MMP9 treatment (*$P < 0.05$, ANOVA; $n = 6$). (*H*) Immunohistochemical staining of HT-29/B6 cells for claudin-1 (red) and occludin (green) without (*Left*) and with (*Right*) MMP9 PEX treatment (representative sample) showed a decrease only in claudin-1 immunoreactivity. All data are mean ± SEM.

leave any motor impairment of pain-related behavior when the isoflurane anesthesia was ended. While rats are an excellent model of perineurial barrier function, the perineurial barrier of mice is rather permeable and axonally released CGRP could pass through into the incubation fluid without prior treatment. Therefore, we did not perform studies in mice or use KO mice. After intraplantar injection of hypertonic saline in rats, the sodium concentration remains only transiently elevated (16), whereas claudin-1 expression in the perineurium is reduced for up to 4 h. Claudin-1 mRNA expression was up-regulated as a compensatory mechanism. Although being beyond the scope of the present study, it would be tempting to investigate the resealing process of the barrier after opening by hypertonic saline. Claudin-1 is a major sealing tight junction protein, because claudin-1 KO mice are not viable because of loss of water through the skin (28). No changes of other tight junction proteins like claudin-5

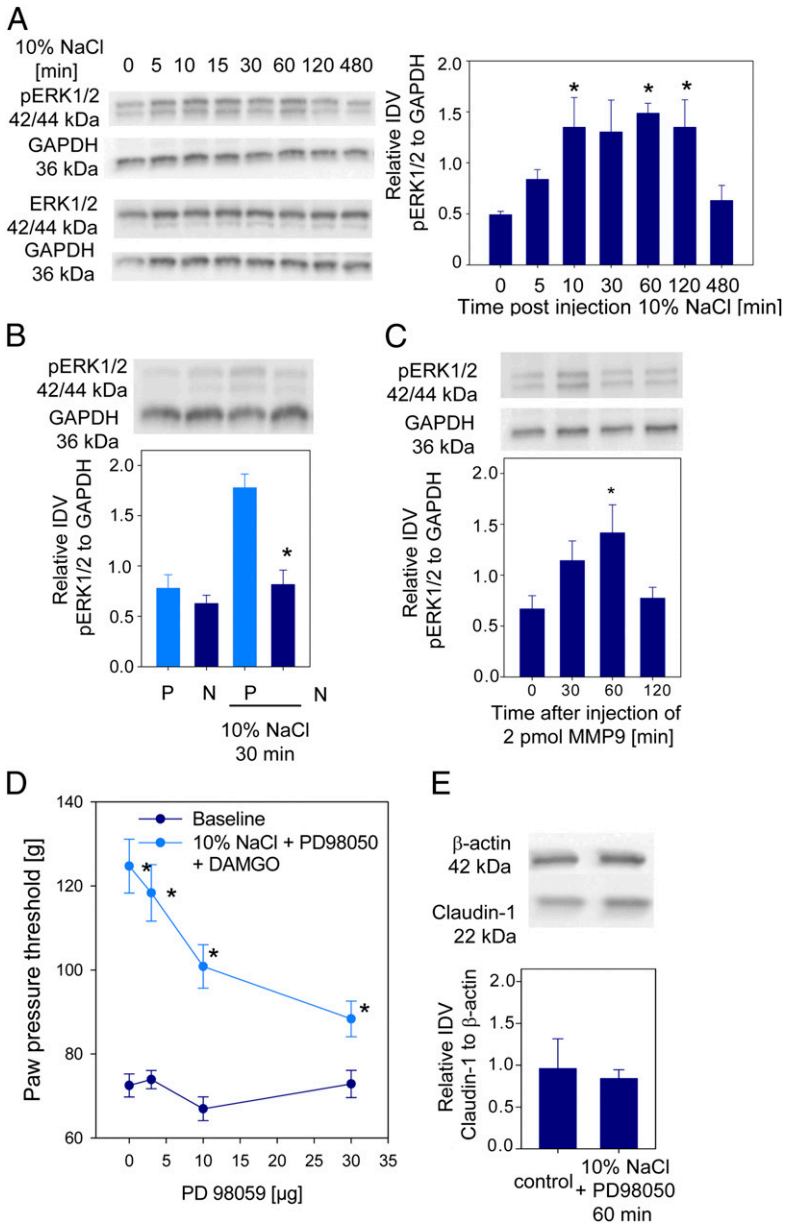

Fig. 7. Hypertonic saline, as well as MMP9 treatment, triggers ERK phosphorylation in the perineurium. (*A* and *B*) Following injection of 10% NaCl, the sciatic nerve cytosolic phosphorylated ERK 1/2 (pERK1/2) and ERK1/2 content in the unseparated nerves was evaluated; pERK increased between 10 and 120 min [relative integrated density value (IDV); $*P < 0.05$, ANOVA; $n = 3$]. (*B*) Perineurium (P) was separated from nerve (N) and pERK-quantified (relative IDV; $*P < 0.05$, ANOVA; $n = 4$), showing increased pERK only in the perineurium only after 10% NaCl treatment. (*C*) pERK1/2 protein expression was analyzed in the cytosolic fraction of sciatic nerve tissue treated without or with 2 pmol of recombinant MMP9 for 30–120 min. (*D*) Mechanical nociceptive theresholds were quantified before (dark blue circles) and 10 min after (light blue circles) injection of 30 μg of DAMGO in 10% NaCl at the sciatic nerve with 10 min of pretreatment with the pERK inhibitor PD98059, which inhibited the effect of 10% NaCl ($*P < 0.05$, ANOVA, Student–Newman–Keuls method; $n = 6$). (*E*) Claudin-1 content remained constant when rats were pretreated with 10% NaCl plus PD98059 compared with control rats (relative IDV; $*P < 0.05$, ANOVA; $n = 3$). All data are mean ± SEM.

and occludin were observed in our study in contrast to nerve crush injury (12). Nevertheless, other tight junction proteins might be involved as well. The crucial role of claudin-1 in barrier formation was confirmed using siRNA knockdown of claudin-1. Opening of the perineurial barrier using hypertonic saline was reversible and functionally restored after 8 h. In preliminary experiments, hypertonic saline treatment induced neither nerve damage nor long-term changes in baseline mechanical nociceptive thresholds.

MMPs play a major role in altering the blood–brain barrier permeability under pathological conditions in vivo (e.g., in focal ischemia) (17). We demonstrated that opening of the perineurial barrier also involves activation of MMP9. MMPs are predominantly expressed in Schwann cells of peripheral nerves under normal conditions and are up-regulated in peripheral nerve injury (29). We now demonstrate that MMPs from three families were expressed in the perineurium under normal conditions. Hypertonic solution appears to trigger the secretion of MMP9 from perineurial cells in line with hypertonic saline-induced release of MMPs from cultured corneal epithelial cells (30) and from neutrophils (31). MMPs effectively degrade other tight junction proteins like occludin (32) or claudin-5 (17). Claudin-1

degradation was demonstrated neither in published studies nor in our own Western blots. However, degradation cannot be completely ruled out because the antibody used might not detect the cleavage products. In our study, we demonstrated that the effects of MMP9 are limited to the regulatory MMP9 PEX domain, which is devoid of the catalytic domain. To support the role of MMP9 PEX further in the opening of barriers, we studied the permeability of the claudin-1–expressing cell line (HT-29/B6). MMP9 PEX treatment reduced the paracellular electrical resistance, increased the permeability for the paracellular marker fluorescein, and reduced the expression of claudin-1.

LRP-1 is not only a receptor for endocytosis of diverse proteins, including MMP9, but a cell-signaling receptor. In Schwann cells, MMP9 PEX binds to LRP-1, promoting migration of these cells (18). LRP-1 has also been implicated in barrier formation of the blood–brain barrier, which is opened after tissue type plasminogen activator treatment mediated by LRP-1 (33). LRP-1 is tethered to the actin network and to focal adhesion sites and controls ERK targeting to talin-rich structures. This pathway could account for claudin-1 redistribution and intracellular degradation after activation of LRP-1 with MMP9 PEX. In our studies, we observed increased ERK1/2 phosphorylation in the perineurium after perisciatic hypertonic saline or MMP9 injection similar to LRP-1 stimulation in Schwann cells (18). In contrast to LRP-1 activation in Schwann cells, no change was seen in Akt phosphorylation. Therefore, the intracellular signaling pathways following LRP-1 activation seem to differ among cell populations.

In summary, we discovered that certain enhancers like hypertonic saline, MMP9 PEX, or claudin-1 siRNA can transiently open the perineurium to allow access for drugs like subtype-selective sodium channel blockers or opioids to provide specific antinociception. This strategy for blocking pain could be advantageous to generate pain-restricted regional anesthesia when preservation of motor responses and nonpainful sensations is desirable. Modulating the tight junction protein claudin-1 could also be considered for treatment of nociceptor-driven chronic pain, such as mononeuropathies or postherpetic neuralgia, if the barrier can be resealed or as an approach for the transfer of genetic material to the peripheral nervous system.

Materials and Methods

A detailed description of the techniques used in this study is provided in *SI Materials and Methods*.

Animals and CFA Inflammation. Animal protocols were approved by the Animal Care Committee of the Senate of Berlin (Landesamt für Arbeitsschutz, Gesundheitsschutz und Technische Sicherheit, Berlin) and the Animal Care Committee of the provincial government of Wurzburg, and they are in accordance with the International Association for the Study of Pain. Male Wistar rats (Harlan Laboratories) weighing 180–220 g were injected under brief isoflurane anesthesia. For the inflammatory pain model, rats were injected intraplantarly with 150 μL of CFA as described previously (34).

Perineurial Injection at Sciatic Nerve. Under brief isoflurane anesthesia, the right sciatic nerve was located using a 22-gauge needle connected to a nerve stimulator (Stimuplex Dig RC; Braun) as previously described (35). A maximum of 300 μL of the indicated substances was injected. Ten percent NaCl (10 g per 100 mL), 0.9% NaCl (0.9 g per 100 mL), MMP9, MMP9 PEX, MMP inhibitors, RAP, PD 98059, and anti-MMP9 antibodies were injected together or 10 min before injection of the active agent as outlined in *Results*.

siRNA Treatment. siRNA (4 μg) was added to polyethylenimine (PEI) [0.45% PEI and 2% dextrose in water (1:5 vol/vol)] and diluted in normal saline. This mixture was applied 48 h, 24 h, and 3 h before coinjection with DAMGO according to the method of Xu et al. (36).

Behavioral Assays. Mechanical paw pressure thresholds (e.g., the suprathreshold external pressure tolerated by the animals before paw withdrawal) were determined using the paw pressure algesiometer (modified Randall–Sellito test; Ugo Basile) (34, 37). Motor function was assessed using a rotarod treadmill (Rota-Rod; Ugo Basile).

Compound Action Potential Electrophysiology and CGRP Release. A sciatic nerve in vitro preparation was used to record A-fiber and C-fiber sensory nerve compound action potentials (38), as well as CGPR release into incubation fluid (19).

Expression and Purification of the MMP9 PEX Domain. The expression plasmid for MMP9 PEX was kindly provided by Martin Roderfeld (Justus Liebig University Giessen, Germany) (20), and it was expressed and purified.

Immunofluorescence and Permeability of the Perineurium. Sciatic nerves from rats were harvested after indicated time points and stained. Claudin-1 immunostaining (1:50 ratio; Invitrogen Immunodetection) was done on paraffin-embedded tissues (39). For MMP or LPR staining, adult rats were perfused with 4% (wt/vol) paraformaldehyde and the sciatic nerve tissue was cryoprotected overnight at 4 °C in PBS containing 10% sucrose as previously described (40). Nerves for permeability evaluation were immersed in 2 mL of EBA (5% bovine albumin labeled with 1% Evans blue; both from Sigma Chemicals) for 1 h (12).

PCR. mRNA of sciatic nerve tissue was semiquantified by Light-Cycler PCR (Roche Diagnostics) as described elsewhere (41, 42).

Western Blotting. Proteins were detected using specific antibodies against claudin-1 (Invitrogen) and Akt, phosphorylated Akt, ERK, and phosphorylated ERK (Cell Signaling) using β-actin (Sigma–Aldrich) or GAPDH (Millipore), respectively, as a protein loading control (39).

Two-Path Impedance Spectroscopy. Electrophysiological analyses were performed on confluent HT-29/B6 cell monolayers (43) mounted in Ussing chambers, and two-path impedance spectroscopy was performed as recently described (44).

Statistical Analysis. All data are presented as mean ± SEM. Data were tested for normality and for equal variance. Multiple measurements were compared by one-way or two-way ANOVA or by one-way ANOVA on ranks in case of data that were not normally distributed. The post hoc comparisons were performed by the Student–Newman–Keuls or Dunnet method. Compound action potential recordings were statistically analyzed by the Wilcoxon signed rank test for repeated measurements in a sample that was not distributed normally. Differences were considered significant if $P < 0.05$.

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1. Binshtok AM, Bean BP, Woolf CJ (2007) Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* 449:607–610.
2. Sagie I, Kohane DS (2010) Prolonged sensory-selective nerve blockade. *Proc Natl Acad Sci USA* 107:3740–3745.
3. Nassar MA, et al. (2004) Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Proc Natl Acad Sci USA* 101:12706–12711.
4. Zimmermann K, et al. (2007) Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures. *Nature* 447:855–858.
5. Schmalhofer WA, et al. (2008) ProTx-II, a selective inhibitor of NaV1.7 sodium channels, blocks action potential propagation in nociceptors. *Mol Pharmacol* 74:1476–1484.
6. Brack A, et al. (2004) Endogenous peripheral antinociception in early inflammation is not limited by the number of opioid-containing leukocytes but by opioid receptor expression. *Pain* 108:67–75.
7. Stein C, Schäfer M, Machelska H (2003) Attacking pain at its source: New perspectives on opioids. *Nat Med* 9:1003–1008.
8. Labuz D, et al. (2009) Immune cell-derived opioids protect against neuropathic pain in mice. *J Clin Invest* 119:278–286.
9. Stein C, Millan MJ, Shippenberg TS, Peter K, Herz A (1989) Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta and kappa receptors. *J Pharmacol Exp Ther* 248:1269–1275.
10. Grant GJ, Vermeulen K, Zakowski MI, Langerman L (2001) Perineural antinociceptive effect of opioids in a rat model. *Acta Anaesthesiol Scand* 45:906–910.
11. Piña-Oviedo S, Ortiz-Hidalgo C (2008) The normal and neoplastic perineurium: A review. *Adv Anat Pathol* 15(3):147–164.
12. Hirakawa H, Okajima S, Nagaoka T, Takamatsu T, Oyamada M (2003) Loss and recovery of the blood-nerve barrier in the rat sciatic nerve after crush injury are

associated with expression of intercellular junctional proteins. *Exp Cell Res* 284(2): 196–210.
13. Pummi KP, Heape AM, Grénman RA, Peltonen JT, Peltonen SA (2004) Tight junction proteins ZO-1, occludin, and claudins in developing and adult human perineurium. *J Histochem Cytochem* 52:1037–1046.
14. Rapoport SI (2000) Osmotic opening of the blood-brain barrier: Principles, mechanism, and therapeutic applications. *Cell Mol Neurobiol* 20:217–230.
15. Antonijevic I, Mousa SA, Schäfer M, Stein C (1995) Perineurial defect and peripheral opioid analgesia in inflammation. *J Neurosci* 15:165–172.
16. Rittner HL, et al. (2009) Antinociception by neutrophil-derived opioid peptides in noninflamed tissue—Role of hypertonicity and the perineurium. *Brain Behav Immun* 23:548–557.
17. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA (2007) Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J Cereb Blood Flow Metab* 27:697–709.
18. Mantuano E, et al. (2008) The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. *J Neurosci* 28:11571–11582.
19. Sauer SK, Bove GM, Averbeck B, Reeh PW (1999) Rat peripheral nerve components release calcitonin gene-related peptide and prostaglandin E2 in response to noxious stimuli: Evidence that nervi nervorum are nociceptors. *Neuroscience* 92:319–325.
20. Roeb E, et al. (2002) The matrix metalloproteinase 9 (mmp-9) hemopexin domain is a novel gelatin binding domain and acts as an antagonist. *J Biol Chem* 277: 50326–50332.
21. Pinto V, Derkach VA, Safronov BV (2008) Role of TTX-sensitive and TTX-resistant sodium channels in Adelta- and C-fiber conduction and synaptic transmission. *J Neurophysiol* 99:617–628.
22. Wilson MJ, et al. (2011) μ-Conotoxins that differentially block sodium channels NaV1.1 through 1.8 identify those responsible for action potentials in sciatic nerve. *Proc Natl Acad Sci USA* 108:10302–10307.
23. Truong W, Cheng C, Xu QG, Li XQ, Zochodne DW (2003) Mu opioid receptors and analgesia at the site of a peripheral nerve injury. *Ann Neurol* 53:366–375.
24. Picard PR, Tramèr MR, McQuay HJ, Moore RA (1997) Analgesic efficacy of peripheral opioids (all except intra-articular): A qualitative systematic review of randomised controlled trials. *Pain* 72:309–318.
25. Huber JD, et al. (2001) Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. *Am J Physiol Heart Circ Physiol* 280:H1241–H1248.
26. Campos CR, Ocheltree SM, Hom S, Egleton RD, Davis TP (2008) Nociceptive inhibition prevents inflammatory pain induced changes in the blood-brain barrier. *Brain Res* 1221:6–13.
27. Dobrogowska DH, Vorbrodt AW (2004) Immunogold localization of tight junctional proteins in normal and osmotically-affected rat blood-brain barrier. *J Mol Histol* 35: 529–539.
28. Furuse M, et al. (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: A lesson from claudin-1-deficient mice. *J Cell Biol* 156:1099–1111.
29. Demestre M, et al. (2004) Characterisation of matrix metalloproteinases and the effects of a broad-spectrum inhibitor (BB-1101) in peripheral nerve regeneration. *Neuroscience* 124:767–779.
30. Luo L, Li DQ, Corrales RM, Pflugfelder SC (2005) Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. *Eye Contact Lens* 31(5):186–193.
31. Chen Y, Hashiguchi N, Yip L, Junger WG (2006) Hypertonic saline enhances neutrophil elastase release through activation of P2 and A3 receptors. *Am J Physiol Cell Physiol* 290:C1051–C1059.
32. Schubert-Unkmeir A, et al. (2010) Neisseria meningitidis induces brain microvascular endothelial cell detachment from the matrix and cleavage of occludin: A role for MMP-8. *PLoS Pathog* 6:e1000874.
33. Polavarapu R, et al. (2007) Tissue-type plasminogen activator-mediated shedding of astrocytic low-density lipoprotein receptor-related protein increases the permeability of the neurovascular unit. *Blood* 109:3270–3278.
34. Rittner HL, et al. (2009) Mycobacteria attenuate nociceptive responses by formyl peptide receptor triggered opioid peptide release from neutrophils. *PLoS Pathog* 5: e1000362.
35. Puehler W, et al. (2004) Rapid upregulation of mu opioid receptor mRNA in dorsal root ganglia in response to peripheral inflammation depends on neuronal conduction. *Neuroscience* 129:473–479.
36. Xu ZZ, et al. (2010) Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. *Nat Med* 16:592–597, 1p following 597.
37. Stein C, et al. (1990) Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. *Proc Natl Acad Sci USA* 87:5935–5939.
38. Kistner K, Zimmermann K, Ehnert C, Reeh PW, Leffler A (2010) The tetrodotoxin-resistant Na+ channel Na (v)1.8 reduces the potency of local anesthetics in blocking C-fiber nociceptors. *Pflugers Arch* 459:751–763.
39. Markov AG, Veshnyakova A, Fromm M, Amasheh M, Amasheh S (2010) Segmental expression of claudin proteins correlates with tight junction barrier properties in rat intestine. *J Comp Physiol B* 180:591–598.
40. Mousa SA, et al. (2007) Nerve growth factor governs the enhanced ability of opioids to suppress inflammatory pain. *Brain* 130:502–513.
41. Brack A, et al. (2004) Mobilization of opioid-containing polymorphonuclear cells by hematopoietic growth factors and influence on inflammatory pain. *Anesthesiology* 100(1):149–157.
42. Brack A, et al. (1997) Glucocorticoid-mediated repression of cytokine gene transcription in human arteritis-SCID chimeras. *J Clin Invest* 99:2842–2850.
43. Amasheh M, et al. (2010) TNFalpha-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFkappaB signaling. *J Cell Sci* 123:4145–4155.
44. Krug SM, Fromm M, Günzel D (2009) Two-path impedance spectroscopy for measuring paracellular and transcellular epithelial resistance. *Biophys J* 97:2202–2211.