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Involvement of TACAN, a mechanotransducing ion channel, in inflammatory but not neuropathic hyperalgesia in the rat

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Abstract

TACAN (Tmem120A), a mechanotransducing ion channel highly expressed in a subset of nociceptors, has recently been shown to contribute to detection of noxious mechanical stimulation. In the present study we evaluated its role in sensitization to mechanical stimuli associated with preclinical models of inflammatory and chemotherapy-induced neuropathic pain (CIPN). Intrathecal administration of an oligodeoxynucleotide antisense (AS-ODN) to TACAN mRNA attenuated TACAN protein expression in rat dorsal root ganglia (DRG). While TACAN AS-ODN produced only a modest increase in mechanical nociceptive threshold, it markedly reduced mechanical hyperalgesia produced by intradermal administration of prostaglandin E_2 (PGE_2), tumor necrosis factor alpha (TNFα) and low molecular weight hyaluronan (LMWH), and systemic administration of lipopolysaccharide (LPS), compatible with a prominent role of TACAN in mechanical hyperalgesia produced by inflammation. In contrast, TACAN AS-ODN had no effect on mechanical hyperalgesia associated with CIPN produced by oxaliplatin or paclitaxel. Our results provide evidence that TACAN plays a role in mechanical hyperalgesia induced by pronociceptive inflammatory mediators, but not CIPN, compatible with multiple mechanisms mediating mechanical nociception, and sensitization to mechanical stimuli in preclinical models of inflammatory versus CIPN.

Keywords

TACAN; Mechanical nociceptive threshold; Hyperalgesia; CIPN

INTRODUCTION

Most clinical pain syndromes are associated with sensitization of nociceptors to mechanical stimuli, especially those pain syndromes associated with inflammation[33,42,44,46,54] and peripheral neuropathy[12,22,31,53]. Mechanotransduction in sensory neurons is a vital process

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underlying several physiological functions, including hearing, touch, pain, and proprioception[14,18,41,61]. The mechanisms underlying mechanotransduction in somatosensory neurons have only recently begun to be elucidated. Piezo2, a mechanotransducing ion channel is now well-established to contribute to the sensation of light touch[34,62], and may also contribute to some forms of nociception[37].

Identified in a screen for transmembrane proteins that underly stretch-activated currents in vascular smooth muscle cells[55], transmembrane protein 120A (Tmem120A), also referred to as TACAN (movement in *farsi*) fulfills criteria for a mechanosensory transduction ion channel[55]. Like Piezos and Transmembrane Channel Like (TMC) proteins[11,36], TACAN shares no sequence homology with other ion channels. TACAN is expressed on mouse dorsal root ganglion (DRG) neurons, predominately in small-diameter DRG neurons, colocalizing with isolectin-B4 (IB4) positivity, a marker of non-peptidergic nociceptors[10,17].

Given that in the setting of inflammatory and neuropathic pain decreased mechanical threshold in nociceptors underlies mechanical hyperalgesia, we evaluated the role of TACAN in mechanical hyperalgesia in multiple preclinical models of inflammatory and neuropathic pain.

METHODS

Animals

Experiments were performed on 220–400 g male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA). Experimental animals were housed three per cage, under a 12-hour light/dark cycle, in a temperature- and humidity-controlled room in the animal care facility at the University of California, San Francisco. Food and water were available *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco, and adhered to the National Institutes of Health guidelines for the care and use of laboratory animals.

Measuring nociceptive threshold

Mechanical nociceptive threshold was quantified using an Ugo Basile Analgesymeter (Stoelting, Wood Dale, IL), to perform the Randall-Selitto paw-withdrawal test. The analgesymeter applies a linearly increasing mechanical force to the dorsum of the rat's hind paw[52,59,60]. Rats were placed in cylindrical acrylic restrainers, with lateral ports that allowed access to the hind paw for nociceptive threshold testing[9]. They were acclimatized to the testing procedure prior to experimental manipulations. Mechanical nociceptive threshold is defined as the force in grams at which a rat withdrew its paw; and baseline threshold was defined as the mean of three readings taken before injection of test agents. To minimize experimenter bias, individuals conducting the behavioral experiments (I.B and D.A) were blinded to experimental treatments; each experiment was performed on a different group of rats. Data are presented as mechanical nociceptive threshold (g).

Drugs

The following drugs were used in this study: low molecular weight hyaluronic acid (hyaluronan) sodium salt from *Streptococcus equi* (LMWH), prostaglandin E_2 (PGE_2), lipopolysaccharide (LPS), and the cancer chemotherapeutic agents paclitaxel and oxaliplatin, all from Sigma-Aldrich (St. Louis, MO); and, rat recombinant TNF-α from R&D Systems (Minneapolis, MN).

PGE_2 was dissolved in absolute ethanol to a concentration of 1 μg/μL, and immediately before experiments diluted in saline. The final ethanol concentration of PGE_2 was ~2%, a concentration previously shown to not affect mechanical nociceptive threshold after intradermal injection[28]. TNFα was first dissolved in phosphate buffered saline (PBS) containing 0.1% BSA, then further diluted in saline before administration. LMWH was dissolved in distilled water to a concentration of 1 μg/μL and further diluted in saline to the desired concentration, at the time of the experiment. PGE_2 (100 ng), TNFα (100 ng) and LMWH (1 μg) were administered intradermally, in a volume of 5 μL, on the dorsum of the hind paw, using a 30-gauge hypodermic needle attached to a 50 μL Hamilton syringe (Hamilton, Reno, NV) by a segment of PE-10 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ). LPS was dissolved in saline to a concentration of 100 μg/kg and administered intraperitoneally (i.p.).

Oxaliplatin- and paclitaxel-induced neuropathy: We have previously described the preclinical models of oxaliplatin and paclitaxel painful chemotherapy-induced peripheral neuropathy (CIPN) used in these experiments[23,26]. Oxaliplatin was freshly dissolved in normal saline at a concentration of 2 mg/mL just prior to intravenous administration (1 mL/kg), via tail vein injection, in rats anesthetized with isoflurane (2.5% in O_2). Paclitaxel was dissolved in absolute ethanol and polyethoxylated castor oil (Cremophor EL; 1:1; Sigma-Aldrich)[1,8,16,20] and diluted in saline, to a concentration of 1 mg/mL, just before intraperitoneal (i.p.) injection[15,32]. Paclitaxel (1 mg/kg) was administered, every other day for a total of 4 doses, in rats anesthetized with isoflurane (2.5% in O_2).

Oligodeoxynucleotides (ODNs) antisense to TACAN mRNA.

The antisense ODN sequence was directed against a unique region of the rat TACAN mRNA.

- Antisense (AS) ODN sequence: 5’-CTT CTT CTG GCG TGT GAT AG-3’
- Mismatch (MM) ODN sequence correspond to the antisense sequence with some bases mismatched (denoted by bold letters): 5’- CT**C** **G**TT CT**T** GC**C** **A**GT GAT A**C**- 3’.

ODNs, synthesized by Life Technologies (Life Technologies, Carlsbad, CA), were reconstituted in nuclease-free 0.9% NaCl and then administered intrathecally, at a dose of 6 μg/μL in a volume of 20 μL, for 3 consecutive days[9]. As described previously[2], rats were anesthetized with isoflurane (2.5% in O_2) and 120 μg of ODN in a volume of 20 μL injected intrathecally using a syringe (300 units/μL) attached to a 29-gauge needle inserted into the subarachnoid space between the L4 and L5 vertebrae. The intrathecal site of injection was confirmed by a sudden flick of the rat’s tail, a reflex that is evoked by subarachnoid space

access and bolus intrathecal injection[43]. Animals regained consciousness approximately 2 minutes after the injection. The use of intrathecal AS-ODN, administered to attenuate the expression of proteins, essential for their role in nociceptor sensitization, is well supported by previous studies by others[47,49,56–58], as well as our group[6,9,13,28,29,48].

SDS-PAGE and Western blotting

To determine the efficacy of the antisense treatment Tmem120A expression in rat lumbar dorsal root ganglia (DRG) was analyzed. Rats were euthanized by exsanguination, while under isoflurane anesthesia, 24h after the last injection of antisense (or mismatch) ODN against Tmem120A mRNA. L4 and L5 DRG were then surgically removed and stored at −80°C until further use. DRGs were transferred into homogenization buffer (100mM NaCl, 1mM EDTA, 2% SDS, 50mM Tris-HCl, pH 7.4) that was supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IL), and manually homogenized with a hand-held plastic pistil. Proteins were solubilized by incubating the homogenized DRG for 2 h at 37°C and 1400 rpm in an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany). Solubilized proteins were extracted from insoluble cell and tissue components by centrifugation for 15 min at 14000 rpm in an Eppendorf tabletop centrifuge. Protein concentration of all samples was determined using the micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as the standard. Mixtures of 60 μg of protein per sample were denatured by boiling in sample buffer [3% SDS, 10% (v/v) Glycerol, 5% (v/v) β-Mercaptoethanol, 0.025 Bromphenol blue, 62.5mM Tris-HCl pH 6.8] for 10 min and electrophoresed on 4–15% pre-cast polyacrylamide gels (Biorad, Hercules, CA) in 25mM Tris containing 192mM glycine and 0.1% SDS. Proteins were electrophoretically transferred to a nitrocellulose membrane using the semidry method [transfer time 2h at 60mA/gel with 47.9mM Tris, 38.9mM Glycine, 0.038% SDS and 20% (v/v) Methanol]. Nitrocellulose membranes were saturated by shaking in antibody dilution buffer [5% BSA in Tris-buffered saline containing 0.1% Tween 20, pH 7.4 (TBST)] for 1h at room temperature, cut in half at ~64kDa and probed with either a rabbit polyclonal anti-Tmem120A antibody (MBS3223965, 1:500, MyBioSource, San Diego, CA) or a rabbit polyclonal anti-PKCε (sc-214, 1:500, Santa Cruz Biotechnology, Paso Robles, CA) antibody, in antibody dilution buffer at 4°C overnight. After rinsing with TBST (3 times at room temperature (RT), 15 min each) both blots were probed with a horseradish peroxidase conjugated anti-rabbit antibody (GE Healthcare Life Sciences, Pittsburgh, PA, 1:2500 in antibody dilution buffer) for 2h at RT. All blotting membranes were rinsed with TBST (3 times at RT, 15 min each) and the immunoreactivities visualized using the West femto chemiluminescence detection system (Pierce Biotechnology). Results were analyzed using computer-assisted densitometry and levels of Tmem120A immunoreactivity were normalized with respect to the PKCε control levels in each sample. The percentage decrease in Tmem120A expression was calculated as: [normalized density for AS/normalized density for MM × 100] −100[3,5].

Statistical analysis

All data are presented as values for mechanical nociceptive threshold (grams) in individual rats. Prism 8.0 (GraphPad Software, San Diego, CA) was used for the graphics and to perform statistical analyses; $P<0.05$ was considered statistically significant. In all behavioral

experiments, the dependent variable was mechanical nociceptive threshold (grams). We used 60 male rats in the behavioral tests. Repeated-measures 2-way ANOVA followed by Bonferroni's multiple comparisons test or Student's *t*-test was used to analyze data.

Our Western blot results are presented as arbitrary units (a.u.), normalized to the reference protein, PKCε. A total of 12 rats were used for this experiment (Fig. 1B). Differences between groups treated with AS- and MM-ODN for TACAN mRNA were analyzed using unpaired Student's *t*-test..

RESULTS

TACAN antisense increases mechanical nociceptive threshold

To provide support for a role of TACAN in mechano-nociception, we measured mechanical nociceptive threshold before and after intrathecal administration of AS- or MM-ODN for TACAN mRNA. Both ODNs were administered once a day for three consecutive days. Mechanical nociceptive threshold (g) was increased when measured 24 h after the last dose of TACAN AS-ODN, and when measured 5 days later, but no longer elevated 8 days later (Fig. 1A). These observations provide support for TACAN as a mechanotransducing ion channel involved in mechanical nociception.

Antisense ODN decreases TACAN in DRG

Western blots of DRG from ODN treated rats demonstrated a 35.6 ± 14.6% (arbitrary units [a.u.] normalized to the reference protein) decrease in expression of TACAN in DRG from AS-ODN compared to MM-ODN treated rats (Fig. 1B). The magnitude of the decrease in TACAN protein is similar to that observed by us[2,9,24,25,48], as well as by other investigators[38,39], for diverse proteins in DRG neurons, following intrathecal administration of AS-ODN against their mRNAs.

TACAN AS-ODN attenuates mechanical hyperalgesia induced by pro-inflammatory mediators

To evaluate the contribution of TACAN to mechanical hyperalgesia induced by pronociceptive inflammatory mediators, groups of rats were treated with AS- or MM-ODN for TACAN mRNA, once a day for three consecutive days. On the fourth day (24 hours after the last injection of TACAN ODN), PGE_2 (100 ng/ 5 μl) (Fig. 2A) or TNFα (100 ng/ 5 μl) (Fig. 2B) was injected intradermally, on the dorsum of the hind paw. Treatment with TACAN AS-ODN markedly attenuated mechanical hyperalgesia induced by both proinflammatory mediators. Hyperalgesia induced by intradermal LMWH (1 μg) was also markedly attenuated in the TACAN AS-ODN-treated group (Fig. 3). Additionally, we evaluated whether TACAN played a role in the hyperalgesia induced by systemically administered LPS (100 μg/kg, intraperitoneal), 24 hours after the third intrathecal administration of TACAN AS- or MM-ODN. Mechanical nociceptive threshold was evaluated 1 hour and 1, 2, 5 and 8 days after LPS. Hyperalgesia induced by LPS was markedly attenuated at 1 hour in the TACAN AS-ODN-treated group, and still attenuated 1, 2 and 5 days later (Fig. 4). These observations demonstrate a major contribution of TACAN to the mechanical hyperalgesia induced by diverse pronociceptive inflammatory mediators.

TACAN AS-ODN does not attenuate mechanical hyperalgesia in chemotherapy-induced peripheral neuropathy (CIPN)

To explore the role of TACAN in the mechanical hyperalgesia associated with the neuropathic pain in cancer patients, in two well-established models of chemotherapy-induced peripheral neuropathy (CIPN), TACAN AS- or MM-ODN mRNA was injected intrathecally, once a day for three consecutive days. Twenty-four hours after the last intrathecal ODN injection, oxaliplatin was administered intravenously (2 mg/kg)[4,23], and mechanical nociceptive threshold evaluated 30 min and 1, 7, 14, 21 and 28 days later. Oxaliplatin-induced hyperalgesia was not attenuated in rats treated with TACAN AS-ODN (Fig. 5).

We next evaluated the role of TACAN in mechanical hyperalgesia associated with paclitaxel CIPN[26]. TACAN AS- or MM-ODN was injected intrathecally, once a day, for three consecutive days. Paclitaxel was administered intraperitoneally (1 mg/kg), 24 hours after the third intrathecal injection of TACAN ODNs, and mechanical nociceptive threshold evaluated 1, 7, 14, 21 and 28 days later. Since paclitaxel was administered every other day, for a total of 4 doses, TACAN AS- and MM-ODNs were also administered every other day after the third consecutive intrathecal administration, until day 6 after paclitaxel (total of 6 doses of ODN). In rats treated with TACAN AS- and MM-ODN paclitaxel-induced hyperalgesia was actually slightly greater in the AS-ODN treated rats, although this difference was not statistically significant at any time point (Fig. 6). These findings support the suggestion that TACAN, does not play a role in chemotherapy-induced neuropathic pain, produced by either oxaliplatin or paclitaxel.

DISCUSSION

TACAN, is a novel ion channel lacking sequence homology to other known classes of ion channels or other proteins[7,21,40], that is blocked by mechanosensitive ion channel toxins[10]. Analysis of its tissue expression revealed that TACAN is expressed in dorsal root ganglion (DRG) neurons, being present in small- to medium-diameter cell bodies, especially in isolectin B4 (IB4)-positive neurons[10], a population of nociceptors that have previously been implicated in mechanical nociception[17], and in tyrosine hydroxylase (TH)-positive neurons. Inducible knockout of TACAN in nociceptors decreased, but did not eliminate, mechanosensitivity and nociceptive behaviors, measured as response frequency to mechanical stimuli[10]. These findings are in line with our results showing that the reversible knockdown of TACAN in nociceptors temporarily increases mechanical nociceptive threshold.

To evaluate the role of TACAN in the *decrease* in mechanical nociceptive threshold associated with inflammation and peripheral neuropathy, we administered an oligodeoxynucleotide antisense (AS-ODN) to TACAN mRNA, intrathecally[2], in preclinical models of inflammatory and neuropathic pain. Intrathecal administration of AS-ODN decreased TACAN protein in DRG. This decrease in TACAN expression was associated with an increase in mechanical nociceptive threshold, providing support that TACAN is a nociceptor mechanotransducing ion channel[10]. Of note, while we did not completely

eliminate TACAN protein in nociceptors, in knockout mice mechanical nociception was also only attenuated but not eliminated[10].

In the present study we sought to determine if TACAN is involved in the decrease in mechanical nociceptive threshold associated with inflammation. In contrast to its modest effect on mechanical nociceptive threshold, TACAN AS-ODN markedly inhibited the hyperalgesia induced by four pronociceptive inflammatory mediators, PGE_2, TNFα, LMWH and LPS. These findings support the hypothesis that TACAN plays an important role in the decrease of mechanical nociceptive threshold, mechanical hyperalgesia, produced by diverse inflammatory mediators.

While our present findings indicate that TACAN contributes to mechanotransduction and the development of mechanical hyperalgesia[10], the magnitude of the attenuation of mechanical hyperalgesia appeared out of proportion to the more modest increase in nociceptive threshold. While this finding is compatible with the involvement of additional mechanotransduction mechanisms in nociceptors, it is not possible to rule out a contribution of other mechanisms to either mechanical nociceptive threshold or mechanical hyperalgesia (e.g., by ligand- or voltage-gated ion channels).

Since hypersensitivity to mechanical stimuli is also a prominent feature of neuropathic pain, we next evaluated whether TACAN also contributes to pain associated with chemotherapy-induced peripheral neuropathy (CIPN), produced by two commonly used clinical chemotherapy agents, oxaliplatin and paclitaxel[26,27,30]. Unexpectedly, treatment with TACAN AS-ODN did not attenuate the mechanical hyperalgesia induced by either oxaliplatin or paclitaxel, indicating that TACAN does not play a substantial role in the mechanical hyperalgesia associated with CIPN. However, given the heterogeneity in the mechanisms underlying the large number of types of neuropathic pain, widely recognized as amongst most difficult pain syndromes to manage, we cannot conclude that TACAN may have or not an effect in other models of neuropathic pain, including CIPN induced by other chemotherapeutic drugs.

We have previously demonstrated that CIPN induced by oxaliplatin is mediated by the IB4-positive population of nociceptors, as it is eliminated by IB4-saporin, a neurotoxin for this class of nociceptors[35]. Since TACAN is strongly expressed in IB4-positive nociceptors, this lack of effect of AS-ODN on the hyperalgesia associated with oxaliplatin CIPN is difficult to explain without invoking an additional class of mechanotransducer in IB4-positive nociceptors. Potential candidate mechanotransducers include degenerin/epithelial sodium channels (DEG/ENaC), transient receptor potential NOMPC-like (TRPN), and Piezo ion channels[50]. With regard to Piezo2, another mechanotransduction ion channel expressed in a subset of DRG neurons that innervate the skin (in low threshold mechanoreceptors)[51], we recently demonstrated that intradermal AS-ODN for Piezo2 mRNA reversed oxaliplatin CIPN[30].

While TRPN channels are implicated in proprioception[19], DEG/ENaC, which regulates turning behavior, may also contribute to mechanical nociception[45]. As Piezo has also been implicated in mechanotransduction required for mechanical nociception[45], different

combinations of ion channels may serve different mechanosensory functions in the same neuron. Additionally, TACAN could co-activate or co-deactivate other ion channels in the cell membrane, leading to hyperpolarization or depolarization of its membrane potential.

TACAN is a TMEM family member. The co-expression of another member of this family, Tmem150C, was found to significantly decrease the apparent activation threshold of Piezo2[7]. The same study also demonstrated that Tmem150C is a regulator of mechano-gated ion channels rather than itself a nonselective ion channel[7]. Thus, we cannot exclude the possibility that TACAN also produces a small decrease in the activation threshold in receptors and may act as a regulator of other mechano-gated ion channels.

In this study we provide support for the recent demonstration that TACAN is a mechanically-gated ion channel that plays a role in mechanical threshold in primary afferent nociceptors 10, and implicate it in mechanical hyperalgesia induced by pronociceptive inflammatory mediators, without contributing to neuropathic mechanical hyperalgesia induced by cancer chemotherapy. While further studies are needed to more precisely define the role of TACAN in nociceptor sensitization and mechanical hyperalgesia, the development of TACAN inhibitors could be a therapeutic target to treat pain, especially in inflammatory conditions.

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Highlights

- Knocking down TACAN in DRG cells increases mechanical nociceptive threshold.
- TACAN plays a crucial role in inflammatory mechanical hyperalgesia.
- Chemotherapy-induced peripheral neuropathy is not dependent on TACAN.

Perspective

We evaluated the role of TACAN, a mechanotransducing ion channel in nociceptors, in preclinical models of inflammatory and chemotherapy-induced neuropathic pain. Attenuation of TACAN expression reduced hyperalgesia produced by inflammatory mediators but had not chemotherapeutic agents. Our findings support the presence of multiple mechanotransducers in nociceptors.

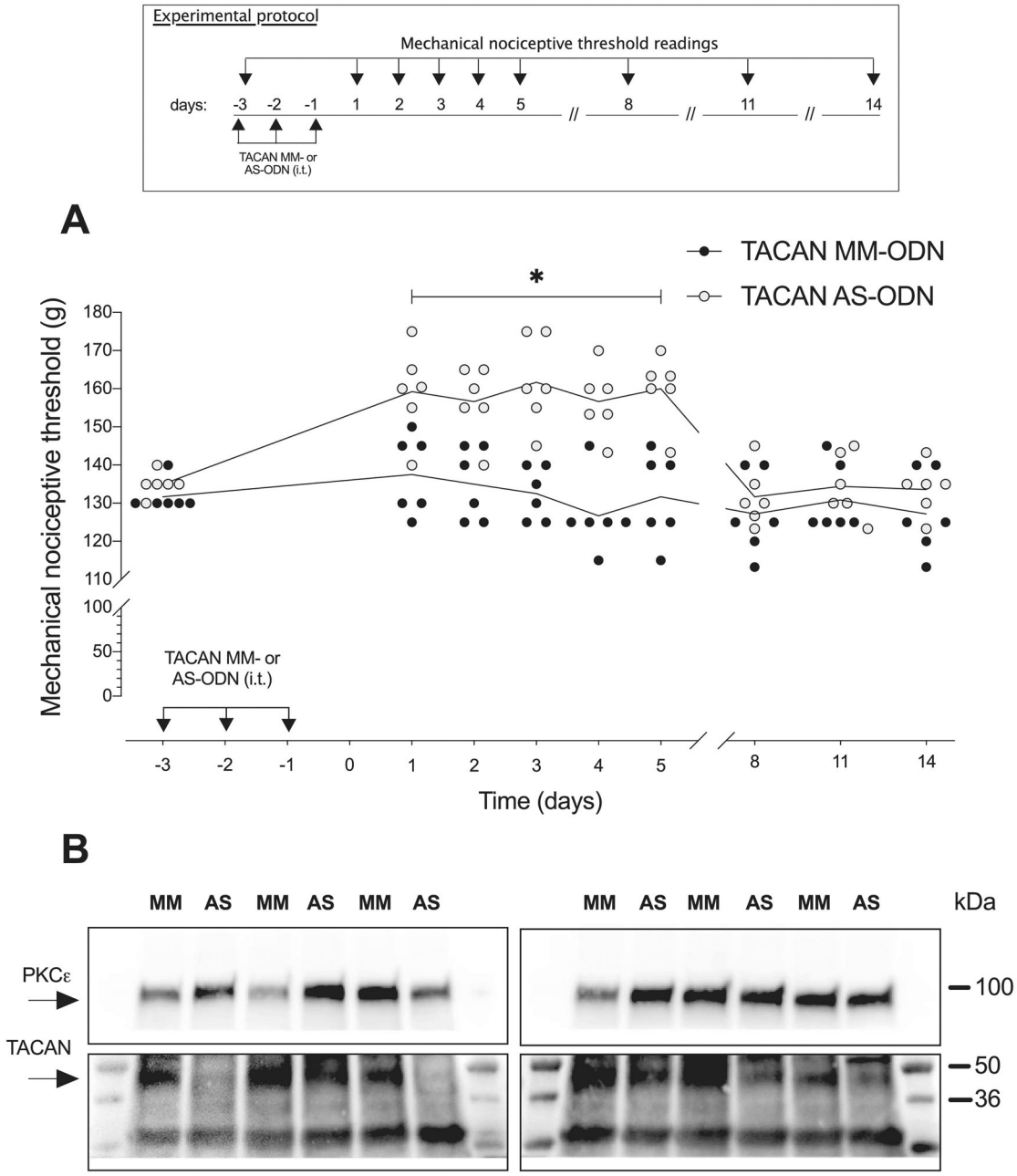

Figure 1. Increase in mechanical nociceptive threshold in rats treated with ODN antisense to TACAN mRNA.

A. Rats were treated intrathecally with AS-ODN (120 μg/20 μL) or MM-ODN (120 μg/20 μL) for TACAN mRNA, once a day for three consecutive days. Mechanical nociceptive threshold was evaluated before the 1st intrathecal administration of AS- or MM-ODN and on days 1, 2, 3, 4, 5, 8, 11 and 14 after its last administration. The group that received TACAN AS-ODN showed an increase in mechanical nociceptive threshold (g) that persisted until 5 days after the last injection ($F_{(8,80)}$=9.219, *p=0.0229 when the TACAN AS-ODN- is compared with the TACAN MM- ODN-treated group; two-way repeated-measures ANOVA

followed by Bonferroni's multiple comparison test). Data in all figures are presented as values for individual animals._n=6 per group.

B. Western blot analysis of DRG extracts from rats injected with 120 μg of antisense ODN/day for three consecutive days revealed a significant decrease in anti-Tmem120A immunoreactivity when compared with the extracts of DRG from mismatch treated rats (−35.6 ± 14.6%, $t_{(10)}$=2.438; *p=0.0175 unpaired student's t-test). The calculated molecular weight of Tmem120A in rat tissue is ~41 kDa (according to UniProtKB database entry Q5HZE2). PKCε, which was used as loading control, has a calculated molecular weight of ~84 kDa (according to UniProtKB database entry P09216). n=6 per group.

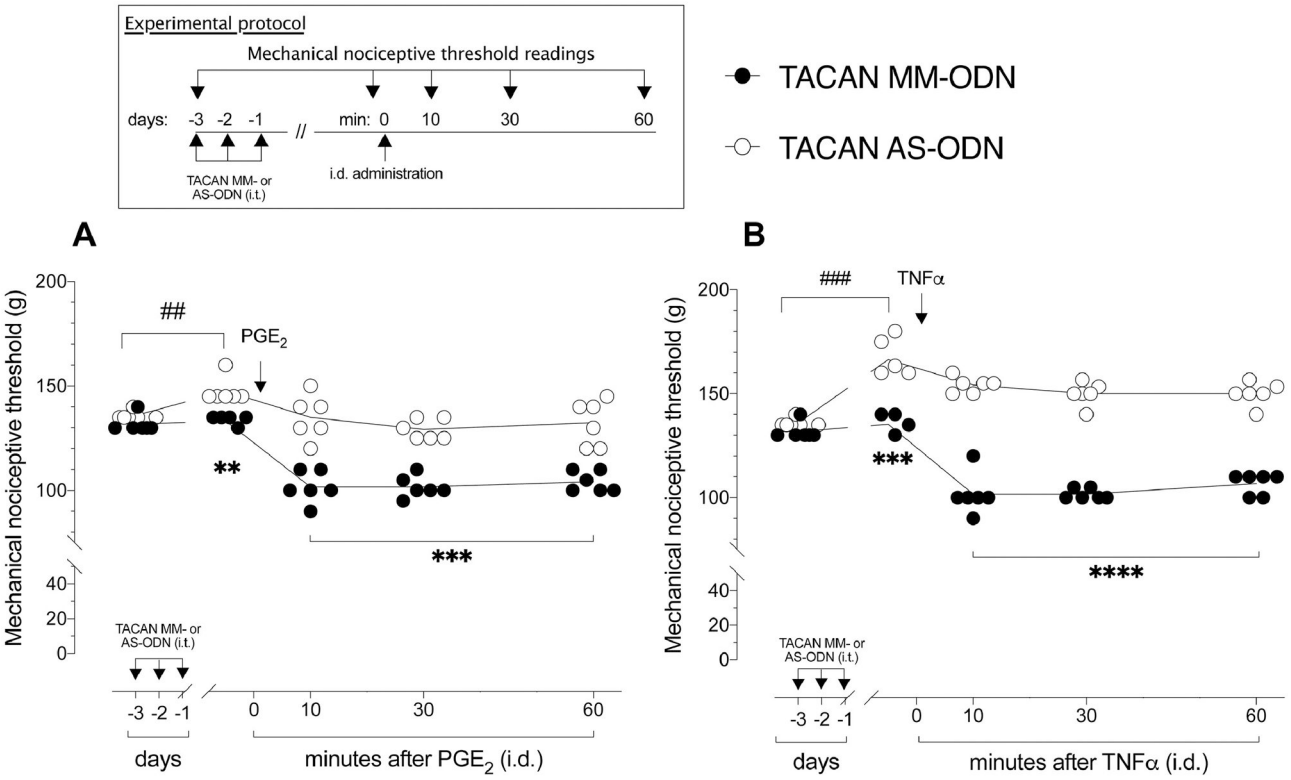

Figure 2. TACAN AS-ODN attenuates mechanical hyperalgesia induced by pronociceptive inflammatory mediators.

A. Rats were treated intrathecally with AS- (120 μg/20 μL) or MM- (120 μg/20 μL) ODN for TACAN mRNA, once a day for three consecutive days. On the fourth day, approximately 24 h after the last intrathecal administration of ODNs, when the mechanical nociceptive threshold was significantly elevated from pre-ODN baseline, in the TACAN AS-ODN-treated group ($t_{(5)}$=1.000; p=0.3632, for the TACAN MM-ODN-treated group and, $t_{(5)}$=7.000; $^{\#\#\#}p$=0.0009, for the TACAN AS-ODN-treated group, when the mechanical nociceptive threshold is compared before and approximately 24 hours after the third intrathecal injection of ODNs; paired Student's t-test), PGE_2 (100 ng/5 μL) was injected intradermally on the dorsum of the hind paw. The mechanical nociceptive threshold was again evaluated 10, 30 and 60 min after PGE_2. The group that received TACAN AS-ODN showed a decrease in PGE_2-induced hyperalgesia when compared to the TACAN MM-ODN-treated group ($F_{(4,40)}$=10.80, $^{**}p$=0.0069, when the TACAN AS-ODN-treated group is compared with TACAN MM-ODN-treated group, before intradermal PGE_2; $^{**}p$=0.0033, when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group 10, 30 and 60 min after PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test). n=6 per group.

B. Rats were treated intrathecally with TACAN AS- (120 μg/20 μL) or MM- (120 μg/20 μL) ODN, once a day, for three consecutive days. On the fourth day, approximately 24 h after the last intrathecal administration of ODN, when the mechanical nociceptive threshold was significantly elevated compared to pre-ODN baseline in the TACAN AS-ODN-treated group ($t_{(5)}$ = 2.072; p=0.0930, for the TACAN MM-ODN-treated group and, $t_{(5)}$=10.07; $^{\#\#\#}p$=0.0002, for the TACAN AS-ODN-treated group, when the mechanical nociceptive

threshold is compared before and approximately 24 hours after the third intrathecal injection of ODNs; paired Student's *t*-test), TNFα (100 ng/5 μL) was injected intradermally on the dorsum of the hind paw. The mechanical nociceptive threshold was again evaluated 10, 30 and 60 min after TNFα. In the group that received TACAN AS-ODN, TNFα-induced hyperalgesia was significantly inhibited compared to the TACAN MM-ODN-treated group, 30 and 60 min after TNFα ($F_{(4,40)}$=38.61, ***p=0.0004, when the TACAN AS-ODN-treated group is compared with TACAN MM-ODN-treated group, before intradermal TNFα; ****p<0.0001 when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group 30 and 60 min after TNFα; two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test). n=6 per group.

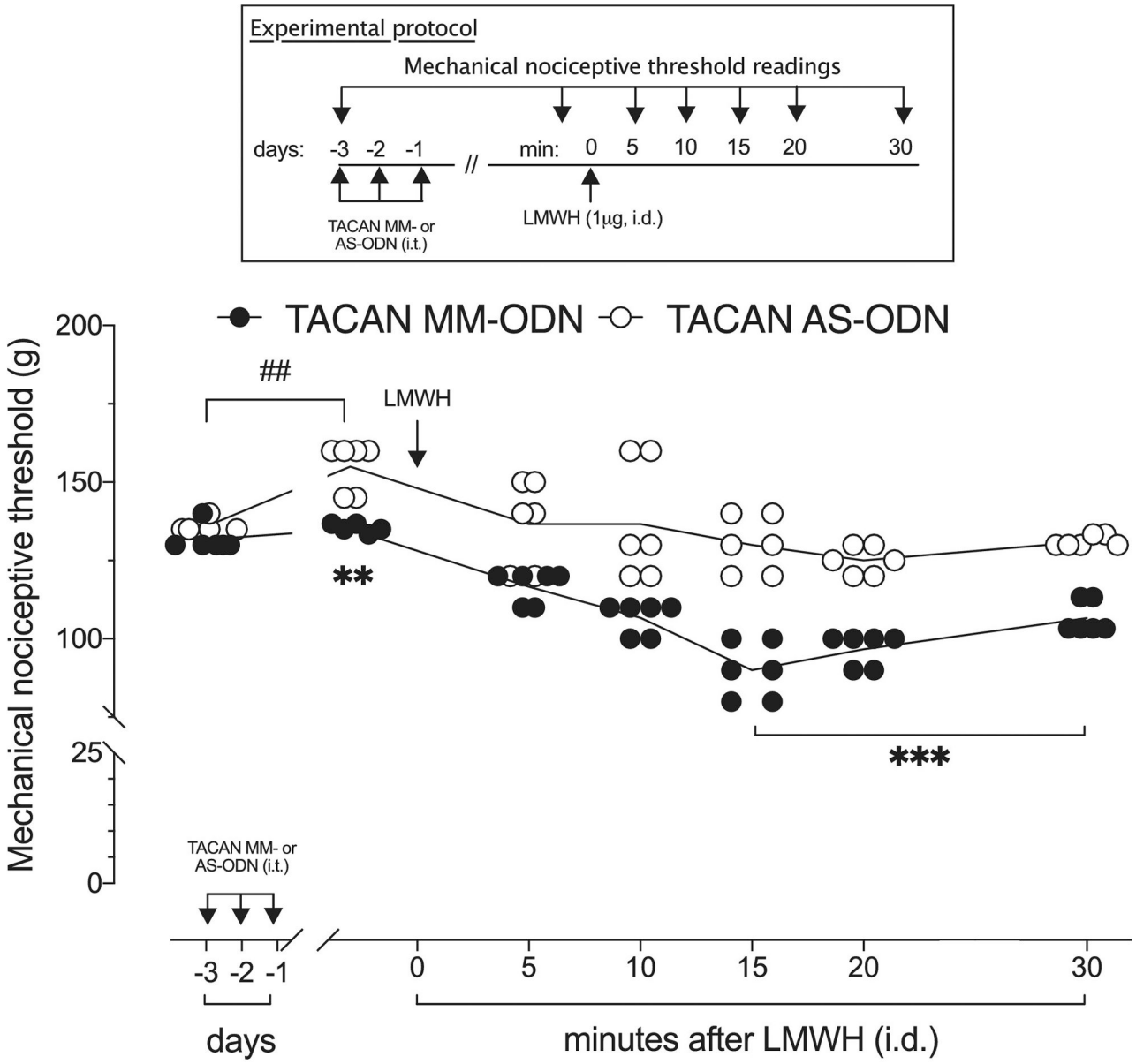

Figure 3. TACAN AS-ODN attenuates LMWH-induced mechanical hyperalgesia.

Rats were treated intrathecally with TACAN AS- (120 μg/20 μL) or MM- (120 μg/20 μL) ODN, once a day, for three consecutive days. On the fourth day, approximately 24 h after the last intrathecal administration of ODN, when the mechanical nociceptive threshold was significantly elevated from pre-ODN baseline in the TACAN AS-ODN-treated group ($t_{(5)}$ = 2.340; p=0.0664, for the TACAN MM-ODN-treated group and, $t_{(5)}$=6.379; $^{\#\#}p$=0.0014, for the TACAN AS-ODN-treated group, when the mechanical nociceptive threshold is compared before and approximately 24 hours after the third intrathecal injection of ODNs; paired Student's t-test), LMWH (1 μg/5 μL) was injected intradermally on the dorsum of the hind paw. Mechanical nociceptive threshold was evaluated 5, 10, 15, 20 and 30 min after LMWH. In the group that received TACAN AS-ODN, hyperalgesia induced by LMWH was markedly attenuated 15, 20 and 30 min after LMWH, when compared with the TACAN

MM-ODN- treated group ($F_{(6,60)}$=5.828, **p<0.0085, when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group before intradermal LMWH; ***p=0.0002 when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group 15, 20 and 30 min after LMWH; two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test). n=6 per group.

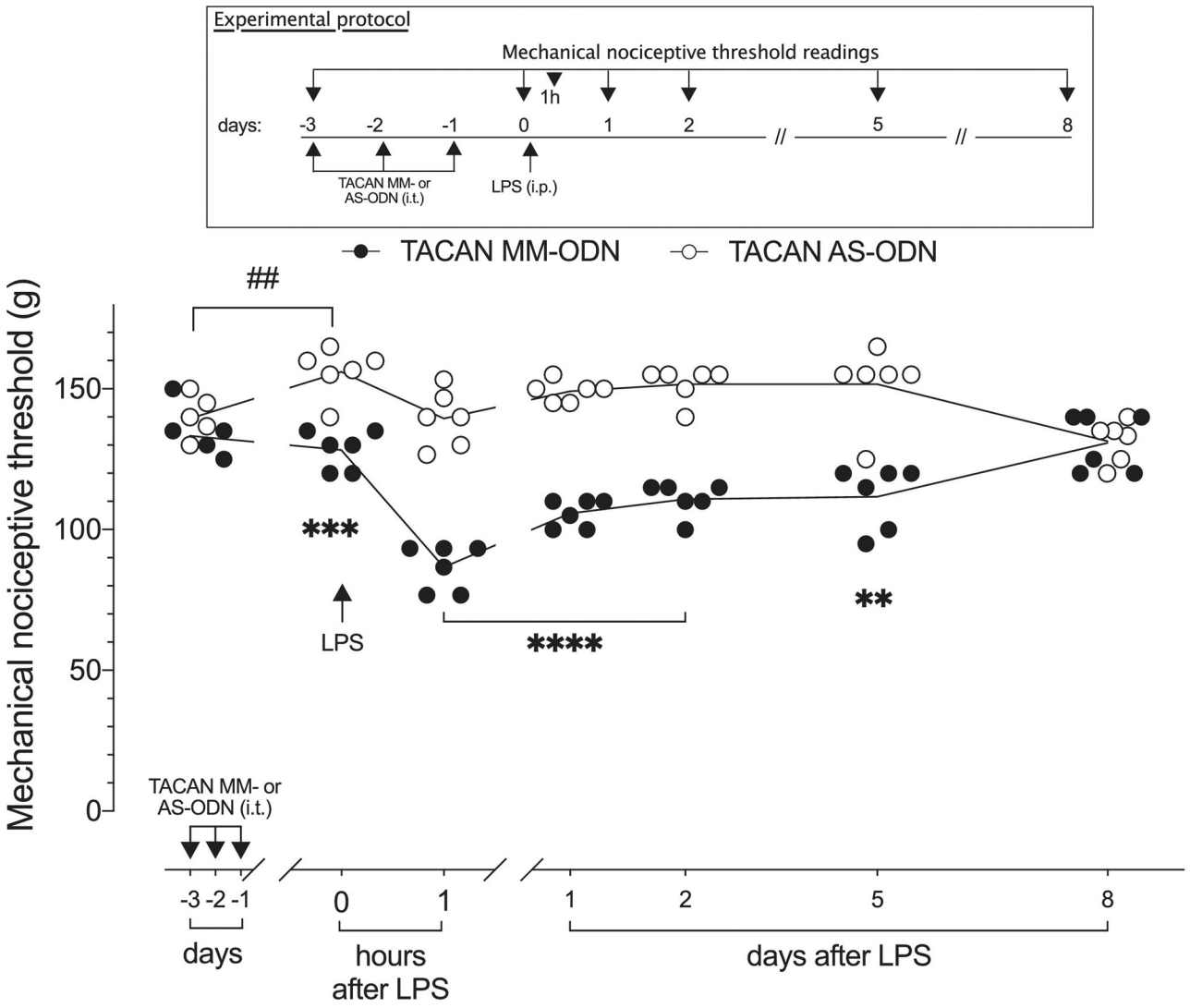

Figure 4. TACAN AS-ODN attenuates LPS-induced mechanical hyperalgesia.

TACAN AS- (120 μg/20 μL) or MM- (120 μg/20 μL) ODN was administered intrathecally in rats, once a day, for three consecutive days. On the fourth day, approximately 24 h after the last intrathecal administration of AS-ODN, when the mechanical nociceptive threshold was significantly elevated from pre-ODN baseline in the TACAN AS-ODN-treated group ($t_{(5)}$=1.291; p=0.2532, for the TACAN MM-ODN-treated group and, $t_{(5)}$=5.534; $^{\#\#}p$=0.0026, for the TACAN AS-ODN-treated group, when the mechanical nociceptive threshold is compared before and approximately 24 hours after the third intrathecal injection of ODNs; paired Student's *t*-test), LPS (100 μg/kg) was injected intraperitoneally (i.p.). The mechanical nociceptive threshold was evaluated 1 h and 1, 2, 5 and 8 days after LPS. In the group that received TACAN AS-ODN, mechanical hyperalgesia induced by systemic LPS was robustly attenuated 1 hour after its i.p. administration, and still attenuated a 1, 2, and 5 days later, compared with the TACAN MM-ODN-treated group ($F_{(6,60)}$=21.25; ***p<0.0009, when the TACAN AS-ODN- is compared with the TACAN MM-ODN-treated group before intraperitoneal LPS; ****p<0.0001 1 h and 1 and 2 day after intraperitoneal

LPS: **p=0.0020; 5 days after intraperitoneal LPS; when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group; two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test). Eight days after i.p. LPS there was no difference between the TACAN AS- and MM-ODN-treated groups. n=6 per group.

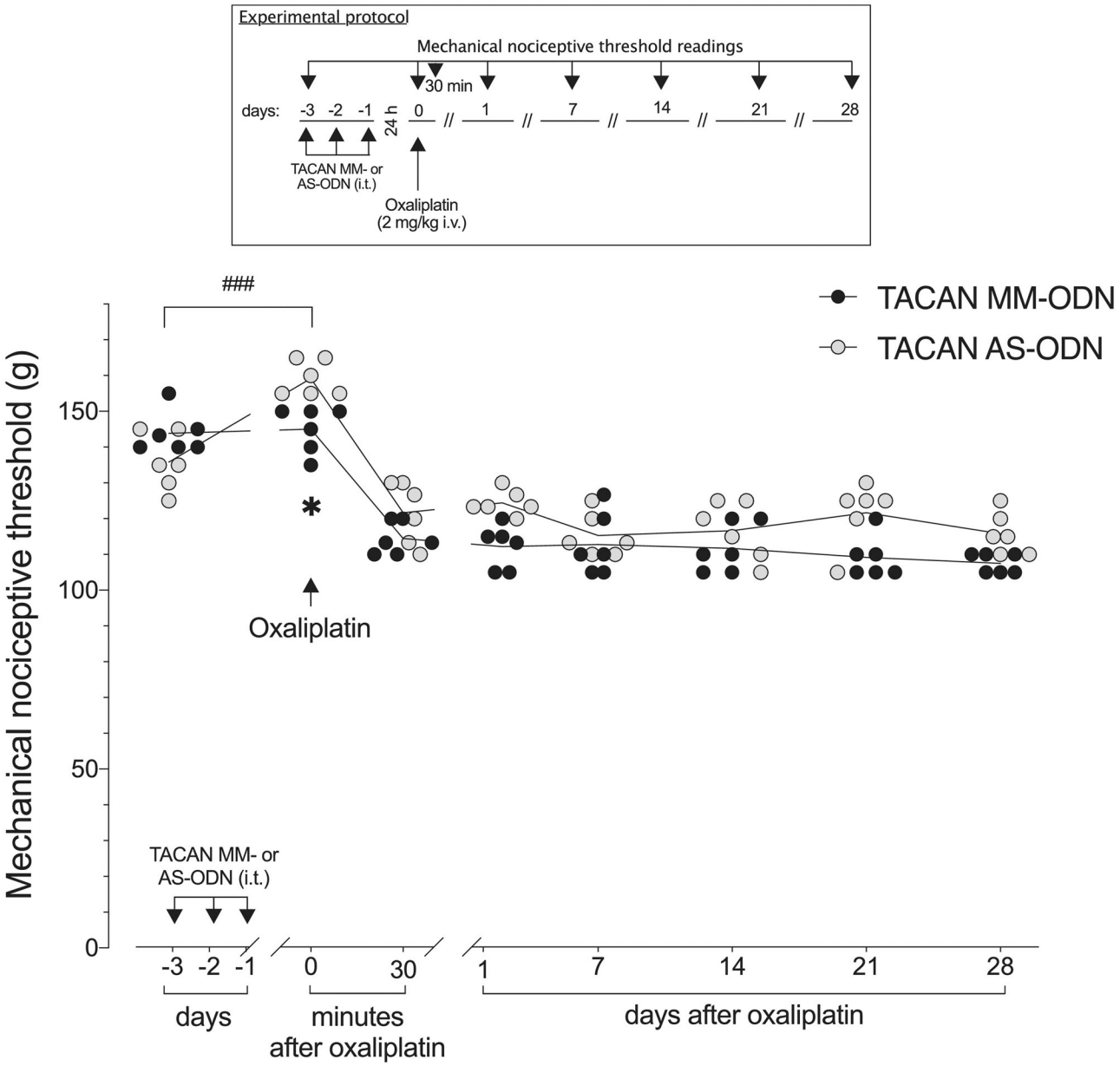

Figure 5. TACAN AS-ODN does not attenuate mechanical hyperalgesia associated with oxaliplatin chemotherapy-induced peripheral neuropathy (CIPN).

Rats were treated intrathecally with TACAN AS- (120 μg/20 μL) or MM- (120 μg/20 μL) ODN, once a day, for three consecutive days. On the fourth day, approximately 24 h after the last intrathecal administration of ODNs, ($t_{(5)}$=0.5218; p=0.6241, for the TACAN MM-ODN-treated group and, $t_{(5)}$=7.593; $^{###}p$=0.0006, for the TACAN AS-ODN-treated group, when the mechanical nociceptive threshold is compared before and approximately 24 hours after the third intrathecal injection of ODN; paired Student's t-test), rats received an intravenous injection of oxaliplatin (2 mg/kg). The mechanical nociceptive threshold was evaluated before the 1st dose of ODNs, before oxaliplatin injection, and then 30min and 1, 7 14, 21 and 28 days after oxaliplatin. TACAN AS-ODN did not attenuate oxaliplatin-induced mechanical hyperalgesia at any time point evaluated ($F_{(7,70)}$=5.874, *p<0.0136, when the

TACAN AS-ODN- is compared with TACAN MM-ODN-treated group before intravenous oxaliplatin; p=0.5868 when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group after oxaliplatin; two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test). n=6 per group.

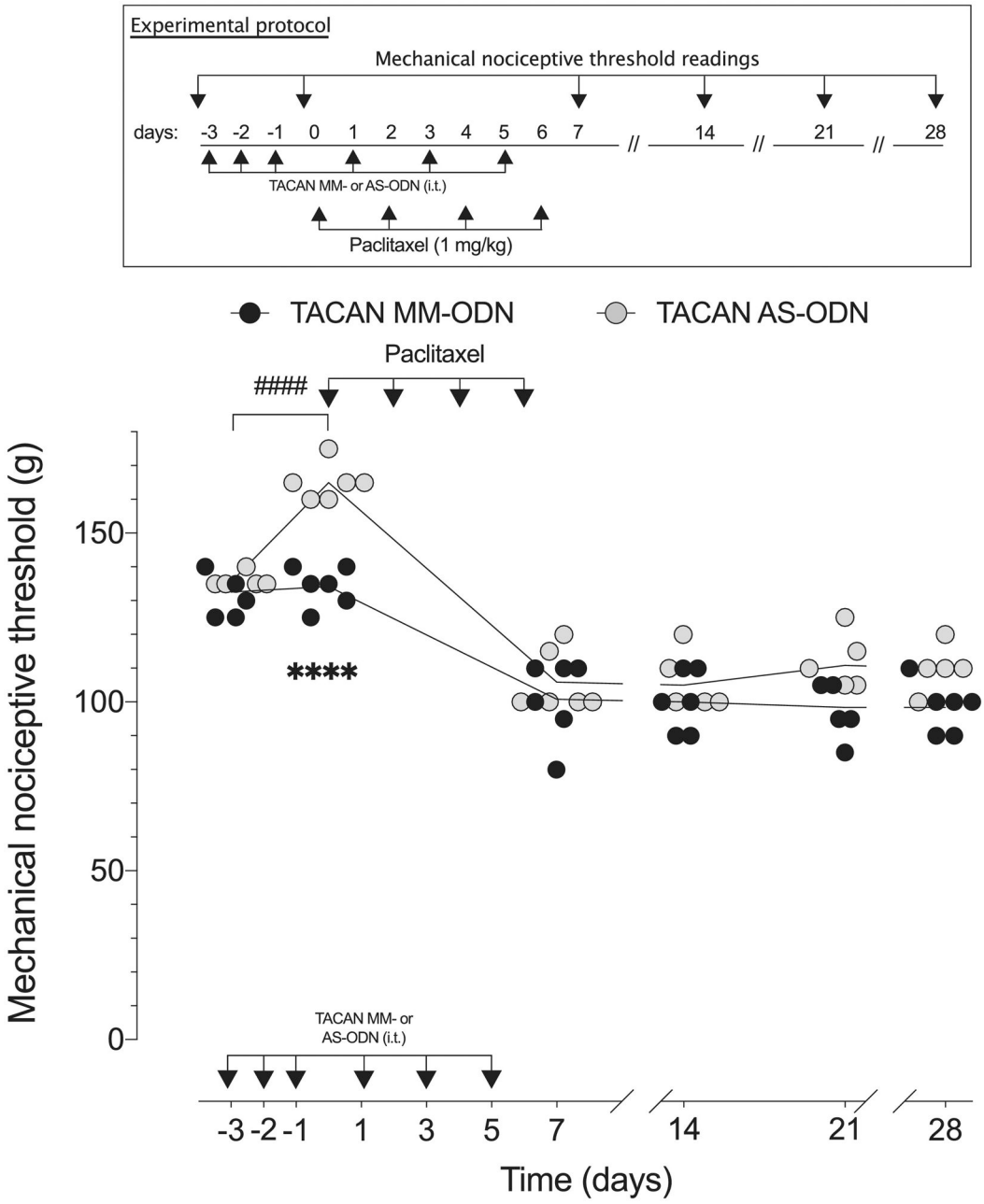

Figure 6. TACAN AS-ODN does not attenuate mechanical hyperalgesia associated with paclitaxel chemotherapy-induced peripheral neuropathy (CIPN).

TACAN AS- (120 μg/20 μL) or MM- (120 μg/20 μL) ODN was administered to rats, once a day for three consecutive days. On the fourth day, approximately 24 h after the third intrathecal administration of ODN ($t_{(5)}$= 0.7906; p=0. 4650, for the TACAN MM-ODN-treated group and, $t_{(5)}$=18.49; $^{\#\#\#\#}p$<0.0001, for the TACAN AS-ODN-treated group, when the mechanical nociceptive threshold is compared before and approximately 24 hours after the third intrathecal injection of ODNs; paired Student's *t*-test), paclitaxel (1 mg/kg) was administered intraperitoneally, every other day for a total of 4 doses (days 0, 2, 4 and 6). TACAN AS- and MM-ODNs were also administered every other day, after the third

consecutive daily intrathecal administration, until day 6 after paclitaxel (total of 6 doses). Mechanical nociceptive threshold was evaluated before the first dose of ODNs, before the first dose of paclitaxel, and then, on days 1, 7, 14, 21 and 28 after the first dose of paclitaxel. TACAN AS-ODN did not attenuate paclitaxel-induced mechanical hyperalgesia at any time point ($F_{(5,50)}$=17.02, ****p<0.0001, when the TACAN AS-ODN- is compared with TACAN MM- ODN-treated group before intraperitoneal paclitaxel; p=0.6081 when the TACAN AS-ODN- is compared with TACAN MM-ODN-treated group; two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test). n=6 per group.