Cutaneous inflammation differentially regulates the expression and function of Angiotensin-II types 1 and 2 receptors in rat primary sensory neurons

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Abstract
Neuropathic and inflammatory pain results from cellular and molecular changes in dorsal root ganglion (DRG) neurons. The type-2 receptor for Angiotensin-II (AT2R) has been involved in this type of pain. However, the underlying mechanisms are poorly understood, including the role of the type-1 receptor for Angiotensin-II (AT1R). Here, we used a combination of immunohistochemistry and immunocytochemistry, RT-PCR and in vitro and in vivo pharmacological manipulation to examine how cutaneous inflammation affected the expression of AT1R and AT2R in subpopulations of rat DRG neurons and studied their impact on inflammation-induced neuritogenesis. We demonstrated that AT2R-neurons express C- or A-neuron markers, primarily IB4, trkA, and substance-P. AT1R expression was highest in small neurons and co-localized significantly with AT2R. In vitro, an inflammatory soup caused significant elevation of AT2R mRNA, whereas AT1R mRNA levels remained unchanged. In vivo, we found a unique pattern of change in the expression of AT1R and AT2R after cutaneous inflammation. AT2R increased in small neurons at 1 day and in medium size neurons at 4 days. Interestingly, cutaneous inflammation increased AT1R levels only in large neurons at 4 days. We found that in vitro AT1R and AT2R acted co-operatively to regulate DRG neurite outgrowth. In vivo, AT2R inhibition impacted more on non-peptidergic C-neurons neuritogenesis, whereas AT1R blockade affected primarily peptidergic nerve terminals. Thus, cutaneous-induced inflammation regulated AT1R and AT2R expression and function in different DRG neuronal subpopulations at different times. These findings must be considered when targeting AT1R and AT2R to treat chronic inflammatory pain.

Keywords: AT1 receptor, AT2 receptor, dorsal root ganglia, inflammatory pain, neuritogenesis, nociceptors.

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Abbreviations used: ABC/DAB, avidin-biotin complex/diaminobenzidine; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; AT1R, angiotensin-II type-1 receptor; AT2R, angiotensin-II type-2 receptor; Azil, azilsartan; CFA, complete Freund’s adjuvant; CGRP, calcitonin-gene-related peptide; DAPI, 4’,6’-diamidino-2-phenylindole; DRG, dorsal root ganglion; GDNF, glial-derived neurotrophic factor; HBSS, Hank’s balanced salt solution; IB4, isolecitin B4; IS, inflammatory soup; Los, losartan; LTM, low threshold mechanoreceptor; MEM, minimum essential medium; NGF, nerve growth factor; PD, PD123319; RRID, research resource identifier (see sci-crcrunch.org); SP, substance P; β-TubIII, beta tubulin III.

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Acute and chronic/pathological pain of peripheral origin results from changes in the activity of primary afferent neurons. Different neuronal subpopulations within the dorsal root ganglia (DRG) are affected by peripheral inflammation causing lowered nociceptive sensory and electrical thresholds in undamaged fibers (Shim et al. 2005; Djouhri et al. 2012) as well as spontaneous pain, mechanical allodynia and hyperalgesia (Djouhri et al. 2006; Pinho-Ribeiro et al. 2017; Cook et al. 2018). Recently, the renin–angiotensin system (RAS) has been shown to be involved in normal and pathological sensory function, including inflammation and pain (Ruiz-Ortega et al. 2001; Suzuki et al. 2003; Bali et al. 2014). Angiotensin-II (Ang-II), which is released during inflammation (Suzuki et al. 2003), interacts with both angiotensin type-I receptor (AT1R) and angiotensin type-2 receptor (AT2R) present in the DRG and other peripheral nervous system structures although their role in sensory pathways has yet to be fully understood (Pavel et al. 2008; Patil et al. 2010; Smith and Muralidharan 2015).

The most widely used antihypertensive drug classes, angiotensin-converting enzyme inhibitors and Ang-II receptor blockers (ARBs), are used to treat inflammatory diseases through actions independent of their effects on blood pressure (Ranjbar et al. 2019). Of notice, AT1R are up-regulated in inflamed tissues. On the other hand, the trend in emerging data supports anti-inflammatory, anti-proliferative and anti-fibrotic roles for AT2Rs, and they also may protect against oxidative stress (Matavelli and Siragy 2015). In fact, it is thought that AT1R and AT2R in general have opposite effects on several cell functions (Chabrashvili et al. 2003; Jones et al. 2008). However, Ang-II activation of AT2R acting through the NF-κB pathway led to pro-inflammatory responses (Wolf et al. 2002; Esteban et al. 2004). These data indicate that the correct functioning of the RAS system demands a complex and finely tuned balance between activation of the two receptors.

The inherent pharmacological consequences of such an integrated mechanism of action are perhaps most clearly seen with Ang-II associated analgesia. Chronic administration of AT1R antagonists has been shown to ameliorate migraine and neuropathic and nociceptive pain (Marques-Lopes et al. 2009; Bali et al. 2014), whereas selective AT2R antagonists have been proposed as a treatment for chronic inflammatory pain and peripheral neuropathy (Smith et al. 2013; Smith and Muralidharan 2015; Shepherd and Mohapatra 2018). It remains unclear whether the beneficial effects of RAS blockers in models of neuropathic pain are mediated directly through action on sensory pathways, or indirectly through their attenuating effects on pro-inflammatory mediators.

There is contradictory evidence regarding the nociceptive effect of activating or blocking the AT2R. For instance, a newly identified natural agonist of AT2R, mycolactone, induces analgesia in patients with Buruli ulcers (Marion et al. 2014). Both AT2R agonists and antagonists can have an anti-nociceptive effect under different circumstances. Evidently, a deeper knowledge of the mechanisms of action of Ang-II on the inflammatory responses, and on the ability of ARBs to decrease pain perception, would clarify these apparently contradictory results. One such mechanism involves the Ang-II release in response to inflammation (Benigni et al. 2010) acting on AT2R to promote neurite outgrowth in cultured cells of neuronal origin (Li et al. 2005; Guimond and Gallo-Payet 2012; Namsolleck et al. 2013) as well as in cultured adult human DRG neurons (Chakrabarty et al. 2008; Anand et al. 2013; Anand et al. 2015). In this respect, evidence suggests that structural plasticity of peripheral sensory innervation may play a main role in pain sensation (Cain et al. 2001).

Prior observations provided indirect pharmacological evidence for the involvement of AT2R in relieving mechanical allodynia after complete Freund’s adjuvant (CFA) injection (Chakrabarty et al. 2013). However, it is unclear what role AT1R and the interaction between AT1R and AT2R play in inflammatory processes, particularly in identified neuronal subpopulations of DRG neurons as they mediate different sensory modalities. To address this, we carried out a phenotypic characterization of the AT1R and AT2R expressing DRG neurons, and examined how their expression changed in these neuronal subpopulations after induction of cutaneous inflammation. We also looked at Ang-II-induced neurotogenesis in DRG neurons in vitro and in vivo, and the relative contributions of AT1R and AT2R activation to this process.

**Materials and methods**

**Animals**

We used 3-month-old female Wistar rats (RRID:RGD_737929; weight range 175–200 g obtained from Servicio de Animales de Laboratorio, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina) for in vivo experiments (initial n = 33) and postnatal day 15 (P15, n = 15) for primary DRG cell cultures. Animals (3 per cage) were housed in large metallic boxes with wire mesh lids under controlled temperature and humidity with 12 h light/darkness cycles and access to food and water ad libitum. All experimental procedures were carried out between 9 and 11 AM. Animals were cared for in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee (CICUAL 31/2014 and 102/2017) of the Universidad Nacional de Cuyo, Mendoza, Argentina.

**Induction of cutaneous inflammation**

Cutaneous inflammation was induced under sevofofourane anesthesia by intradermal injection of 100 µL CFA (Sigma, St Louis, MO, USA, Cat# F5881) as previously described (Haskins et al. 2017). To examine the expression of AT1R and AT2R, DRG were removed after 1 day (CFA1, n = 5 rats) and 4 days after CFA (CFA4, n = 5 rats). Control rats (n = 5) received a single injection of 100 µL sterile saline solution. Animals were allocated to each experimental group using a random sequence generator (https://www.random.org/sequences/). Rats were numbered from 1 to 15, and then assigned to
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one of three groups labeled A, B, and C. The software essentially assigned a rat (via its identifier number) to each group until all had five rats each. Notice that a randomized sequence does not contain duplicates (Kim and Shin 2014). No exclusion criteria were predetermined and no animals died during the experiments. Because the CFA treatment can cause mild to moderate pain, we observed the behavior of the rats twice a day looking for signs of significant discomfort (like failure to groom etc.). We also weighed them daily and measured their consumption of water and food. Any rats exhibiting a 10% weight loss or a 20% drop in water/food intake would be terminated by humane culling. This, however, was not needed. Analgesia was not provided as most analgesics have anti-inflammatory activity or are opioids, and both will interfere with the aims of the study.

Tissue preparation

After treatment, rats were anesthetized with ketamine (Cat# 01750020010) plus xylazine (Cat# 01750020170, both from Richmond VetPharma) (50 mg/kg & 10 mg/kg) i.p. and transcardially perfused with saline supplemented with heparin (10 U/ml, Sigma, Cat# H3393) followed by Zamboni’s fixative (Stefanini et al. 1967). Next, skin, L5 DRGs, spinal cord, dorsal root, spinal, and sciatic nerve were dissected and post-fixed for 20 min in Zamboni’s at RT and dipped in sequentially through graded sucrose solutions (from 10 to 30%) at 4°C. Tissue was frozen and kept at −80°C until used. Serial 7 µm cryostat sections were cut for all tissues except skin (10 µm) and spinal cord (12 µm). Sections were placed on gelatin-coated slides and stored at −20°C for subsequent immunohistochemistry.

Primary antibodies against AT1R and AT2R

We used a goat anti-AT2R from Santa Cruz Biotechnology, Santa Cruz, CA, USA (Cat# sc-48452, K15, RRID: AB_2225720, 1 : 200) also used in the DRG by (Anand et al. 2013) and other cell types (Dolley-Hitze et al. 2010). We chose this antibody because of concerns about the selectivity of other commercially available antibodies against AT2R (Haefke et al. 2013). A polyclonal rabbit anti-AT1R (Santa Cruz Biotechnology Cat# sc-579, RRID: AB_2225713, 1 : 200) was used (Butler et al. 2017). We performed a further characterization of these antibodies comparing them against two other commercially available anti-AT2R antibodies (Figure S1). To do this, we ran Western blot analysis and found that AT1R (306) and AT2R (K-15) both detect a single band of the predicted molecular weight in DRGs of three different ages and also in the spinal cord (A-B). However, anti-AT2R (H-134) marked a large number of additional bands (C). Next, we examined how the antibodies performed at staining adrenal gland, a known physiological target for Ang-II where the distribution of AT1R and AT2R expression has been reported using non-immune methods. We found that AT1R (306) and AT2R (K-15) but not Alomone’s AAR-012 selectively stained the zona glomerulosa and cells within the adrenal medulla as expected (Johren et al. 1995; Macova et al. 2008; Pavel et al. 2008). We found that ATIR (306) selectively stained motoneurons in the ventral horn of the spinal cord (Pavel et al. 2008), whereas AT2R (K-15) was remarkably more selective than Alomone’s AAR-012 staining DRG neurons. K-15 also stained cortical neurons, in agreement with reports using in situ hybridization (Lenkei et al. 1997).

Other primary antibodies and IB4-binding

We used phenotypic markers of neuronal subpopulations: (i) Staining with isolectin B4 (IB4, Cat# B-1205, RRID: AB_2314661, 1 : 400), which labels small, C- neurons (Fang et al. 2006), (ii) Mouse anti-NF200 (clone RT97, RRID: AB_528399, DSHB, Iowa University, 1 : 4000). (iii) Rabbit anti-TrkA (Millipore Corporation, Bedford, MA, USA, Cat# 06-574, RRID: AB_310180, developed by L. Reichardt; 1 : 1000). (iv) Guinea pig affinity purified anti-Substance P from Neuromics (Cat# GP14110, RRID: AB_2315368, 1 : 500). (v) Rabbit anti-CGRP (1 : 2000) characterized by (Gibson et al. 1984) and validated by (Lawson et al. 1996). A mouse anti-β-tubulin III (β-TubIII) antibody was also used (clone SDL_3D10, Cat# ab11314, RRID:AB_297918, 1 : 1000). A rabbit anti-PGP9.5 was also used to estimate total neurite length in vitro (Cat# RA12103, NeuroMics, RRID: AB_2315126, 1 : 400).

Western blotting

Western Blots were performed as previously described (Acosta et al. 2014; Benitez et al. 2017) (Figure S1). Total protein was extracted from whole L4/L5 DRGs and spinal cord using Laemml buffer supplemented with a protease/phosphatase inhibitor cocktail (HALT, Cat# 87786, Thermofisher, Waltham, MA, USA). Samples of ∼10 µg of total protein were run on 8% polyacrylamide gels and transferred to PVDF membranes (Cat# GE10600023, Amersham Pharmacia Biotech, Piscataway, NJ, USA) before blotting. Membranes were blocked with 5% semi-skinned milk dissolved in tris-buffer saline (TBS) during 1 h at RT and then probed overnight with 1 : 500 polyclonal rabbit anti-AT1R (306) or 1 : 1000 goat anti-AT2R (K-15) or 1 : 1000 rabbit anti-AT2R (H-143). We used 1 : 4000 mouse anti-α-tubulin (eBioscience, Cat# 14–4502–80, RRID:AB_1210457) as loading control. The membranes were washed three times for 5 mins with 0.1% Tween 20 in TBS (TBST) and then blocked again with 5% semi-skinned milk for 30 min before incubation for 2 h at RT with either 1 : 2000 peroxidase-labeled anti-rabbit (Cat# PI-1000, RRID:AB_2336198) or anti-goat (Cat# PI-9500, RRID:AB_2336124) both from Vector Laboratories. The membranes were washed three times for 5 min with TBS and twice for 5 min with TBS. Protein bands were developed using ECLPlus (Amersham) and visualized with the LAS-4000 System (Fujifilm).

Immunohistochemistry

Avidin-biotin complex immunohistochemistry was carried out following previously published protocols (Acosta et al. 2014). Sections were incubated in primary antibody (anti-AT2R 1 : 200 or anti-AT1R 1 : 200) diluted in 0.05% Triton X-100 in PBS buffer (PBST) overnight at 4°C. On the following day, sections were incubated with biotinylated horse anti-goat (Cat# BA-9500, RRID: AB_2336123) or anti-mouse (Cat# BA-2001, RRID: AB_2336180) antibodies (1 : 400) (Vector Laboratories, Burlingame, CA, USA) for 30 min at 20-22°C. After washings with PBST, sections were incubated 30 min with Streptavidin-HRP (1 : 200, Cat# S5512, Sigma). To develop the reaction, an ABC standard kit from Vector Laboratories was used (Cat# PK-4000, RRID:AB_2336818). For comparisons between different treatments, all sections were incubated simultaneously for the same length of time.

Double/triple immunofluorescence was conducted as previously described (Benitez et al. 2017). Tissues were incubated sequentially in the first primary antibody overnight at 4°C and in secondary
antibody appropriate for that primary antibody or IB4 for 2 h at 20-22°C. Anti-AT2R was used at 1:200, anti-AT1R at 1:200, anti-TrkA at 1:1000, anti-NF200 at 1:4000, anti-Substance P at 1:500, anti-β-TubIII at 1:1000 and anti-CGRP at 1:2000. Secondary antibodies were 1:400 donkey anti-rabbit Alexa-488 (Cat# A32790, RRID:AB_2762833) or Alexa-594 (Cat# A32754, RRID:AB_2762827), goat anti-rabbit Pacific blue (1:100, Cat# P_10994, RRID:AB_2539814), 1:400 goat anti-mouse Alexa-488 (Cat# A_21141, RRID:AB_2535778) or Alexa-594 (Cat# A_21145, RRID:AB_2535781), 1:400 goat anti-guinea pig Alexa-594 (Cat# A_11076, RRID:AB_2534120) and donkey anti-goat Alexa-488 (Cat# A32814, RRID:AB_2762838) or Alexa-594 (Cat# A32758, RRID:AB_2762786), all from Invitrogen. IB4 was conjugated with either FITC or DyLight 594 (1:500, Vector Laboratories, Cat# DL_1207).

**Cell cultures**

DRGs from all rostrocaudal levels from P15 rats, one per culture were dissected out following previously detailed protocols (Acosta et al. 2012; Acosta et al. 2014). We choose this age because (i) the neurons have a better survival profile than adult neurons; (ii) they attached well to a collagen substratum unlike older, adult DRG neurons and (iii) we have already demonstrated that they express levels of AT2R similar to that of adult DRG (Benitez et al. 2017). Briefly, the ganglia were enzymatically digested at 37°C in 0.625% trypsin (Cat# 27250018) for 25 min and then with 0.5% collagenase (Cat# 17100017) for 20 min in HBSS (Hank’s Balanced Salt Solution) subsequently quenched with DMEM/F12 (Cat# 11320033) plus 5% fetal bovine serum (Natocor, Argentina) and mechanically dissociated. Isolated cells were plated onto round coverslips (Bellco Glass, Germany) previously coated with rat tail collagen (300 ng/mm²). Neurons were allowed to attach to the substratum for 3–4 h before addition of DMEM/F12 supplemented with N2 medium (Cat# 17502001) and penicillin/streptomycin (Cat# 95950141). Cells were kept at 37°C in a 5% CO₂ incubator.

**In vitro treatment with inflammatory soup**

Three cultures (from 3 rats) were divided in two groups: one group with no trophic factors (NTF) or for 1 day in vitro (DIV) with mouse nerve growth factor (NGF) 7S (10 ng/mL, Cat# 450-01) or rat recombinant glial-derived neurotrophic factor (GDNF) (20 ng/mL, Cat# 450-10) (Peprotech EC, London, UK). We chose NGF as AT2R+ and ATIR + neurons co-localized with trkA (the high-affinity receptor for NGF). As most AT2R+ neurons also bind IB4, we tested GDNF, the trophic factor on which IB4 + neurons depend (Dubin and Patapoutian 2010). The other group received in addition an inflammatory soup (IS) containing: 10 μM histamine (Cat# H7125), 10 μM α,β-methylene ATP (Cat# M6517), 1 μM prostaglandin-E2 (Cat# P0409), 1 μM bradykinin (Cat# B3235), 10 μM serotonin (Cat# H9523), 100 nM IL-6 (Cat# 10406), 1 μM TNF-α (Cat# T5944), and 1 μM substance P (Cat# S6883). All drugs obtained from Sigma. These are referred to as NTF + IS, NGF + IS, and GDNF + IS.

**In vitro treatment with Ang-II**

Another three cultures (from 3 rats) received daily doses of NGF 7S (10 ng/mL), Ang-II (20 nM, Cat# GPA-100) alone or in combination with AT1R/AT2R selective blockers: 10 nM azilsartan (Cat# A-245) and/or 50 nM PD123319 ditrifluoroacetate (Cat# P-245, all from Alomone Labs, Israel). The cultures were pre-incubated for 15 min at 37°C with the antagonists before addition of Ang-II. Control cultures received neither trophic factors nor Ang-II. Neurons were treated for 2 days (2DIV) to allow measurement of neurite outgrowth.

**Semi-quantitative RT-PCR**

Specific mRNA levels from three cultures with or without IS were determined by RT-PCR as previously described (Marsh et al. 2012; Kunda et al. 2014; Benitez et al. 2017). mRNA was extracted using RNeasy (Cat# 74104, Qiagen, Valencia, CA, USA). Total mRNA was quantified using a Nanodrop and only samples with an A260: A280 ratio above 1.8 were used. cDNA was synthesized from 200 ng total RNA using the M-MLV kit (Cat# 9PIM170, Promega, Madison, WI, USA) supplemented with RNaseOut (Cat# 1077719, Thermo Fisher). AT2R and AT1R mRNA were amplified using Taq polymerase (InbioHighway, Argentina). For AT2R the program consisted of 2 min at 94°C, 34 cycles of 20 s at 94°C, 20 s at 60°C, and 45 s at 72°C, followed by 5 min at 72°C as final extension. For AT1R the program was 3 min at 94°C, 34 cycles of 1 min at 94°C, 1 min at 58°C, and 45 s at 72°C, followed by 15 min at 72°C. β-actin mRNA was used as loading control (32 cycles). We performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination.

Primers sequences were as follows: AT2R forward: 5’-AACCTTCAGTTGTGTGCACC-3’; reverse 5’-CAGGCTAAAAAGGAGCCGATCATC-3’, predicted product size 335 bp. ATIR forward: 5’-ATCCAAAGATGCTGACCCAAA-3’, reverse 5’-GCCAAGGCCAGTAGGGAAG-3’, predicted product size 179 bp. β-actin forward: 5’-CGGACCCCTGTTGCC-3’, reverse 5’-ACCCACACTGTGCACATCA-3’, predicted product size 290 bp. All primers were synthesized by Ruralex (Argentina), custom-designed and checked for specificity using Primer-BLAST. Images of the RT-PCR SYBR Safe (Cat# S33102, Thermo Fisher) stained agarose (Cat# A9539, Sigma) gels were acquired with the LAS-4000 system (Fujifilm) and quantified with ImageJ software (Benitez et al. 2017).

**Immunocytochemistry**

Cultures were removed from the incubator, washed two times with PBS and then fixed in 4% PFA (Cat# P6148, Sigma) with 4.2% sucrose (Biopack, Argentina) for 20 min at 4°C, washed again with PBS and permeabilized with 0.2% v/v Triton X-100 (Biopack, Argentina). Non-specific binding sites were blocked by 1 h incubation at 20-22°C with 5% BSA. Triple immunofluorescence was performed as previously reported (Acosta et al. 2012; Acosta et al. 2014). Coverslips were incubated sequentially in the first primary antibody overnight at 4°C and then in fluorescent secondary antibody appropriate for that primary antibody for 1 h at 20-22°C. This cycle was repeated for each primary antibody. Coverslips were mounted with Mowiol 4–88 (Cat# 475904, Merck). Primary antibodies were applied following the sequence: mouse anti-β-TubIII (1:500), goat anti-AT2R (1:200), and rabbit anti-AT1R (1:200). Secondary antibodies were donkey anti-mouse DyLight 549 (1:400, Vector Labs, Cat# A17828), donkey anti-goat DyLight 488 (1:200, Vector Labs, Cat# SA5-10086, RRID:AB_2556666), and goat anti-rabbit Pacific Blue (1:200).
In order to examine the role of AT1R and AT2R on *in vivo* neuritogenesis we divided 18 rats into three experimental groups (see Fig. 1). Rats were allocated to each experimental group using the same procedure described above (see Induction of cutaneous inflammation) but starting up with 18 numbered rats. One group (*n* = 6) was given oral Losartan (potassium salt, Cozaar, MSD) dissolved in their drinking water (100 mg/l). The treatment lasted 4 weeks. The amount of daily drug ingested by each rat was calculated based on the total volume of water drank divided by the number of rats in each cage. This rendered an effective average dose of losartan of 20 mg/Kg/day. This dosage and length of treatment was used elsewhere to examine numerous physiologically relevant processes (Bayar et al. 2015; Kamber et al. 2015; Akershoek et al. 2017). The second group (*n* = 6) received oral losartan as the first group, but from day 25 to 28 also received a daily i.p. injection of PD 123319 (1mg/Kg/day (Sanchez et al. 2008)). Our aim was to assess the effect on neuritogenesis of acutely antagonizing AT2R in rats where AT1R was already suppressed. Control group (*n* = 6) received normal drinking water (NDW). On day 28, cutaneous inflammation was induced in all rats as described above.

**Image acquisition and analysis**

All analyses were performed as previously detailed in (Acosta et al. 2014; Benitez et al. 2017) on images captured on a Nikon 80i microscope and the quantification was performed using HCImage (Hamamatsu, RRID:SCR_015041). For each experimental comparison, four to six fields were captured at 40X with identical settings during the same session. All intensity measurements were carried out at the soma as it is assumed that soma levels are similar to those present at the nerve terminals. This quantitative analysis was the only one performed blindly.

**AT2R-AT1R intensities measurement**

Only neurons with visible nuclei from mid-sections of L5 DRGs were measured. The cross sectional area and mean whole cytoplasmic, edge and inner pixel density were determined for each neuron. We used cytoplasmic levels as indicative of the total available protein including newly synthesized receptors. In the case of the whole cytoplasm, nuclei were excluded. For edge we drew a line (thickness 3 pixels) over the cell perimeter, that likely reflects the amount of the receptor close to or in the plasma membrane). For the perinuclear intensity we also drew a line over the region surrounding the nucleus (from here on inner) (Figure S2). In both cases, the software averages the pixel density (as mean gray) for the entire region under the line. We have used a similar approach to measure membrane-associated (edge) and inner staining for TREK2 (Acosta et al. 2014), α3-Na+/K+-ATPase (Parekh et al. 2010), and HCN1/HCN2 (Acosta et al. 2012) in the DRG. The mean pixel densities for each region of the five most intensely stained and the five least intensely stained neurons in the analyzed section provided 100% and 0% values, respectively. All pixel densities were converted to % intensities as described in (Acosta et al. 2014; Benitez et al. 2017; Haskins et al. 2017). Neurons were classed by size: small (those with areas < 400 μm²), medium (400 to 800 μm²), and large (> 800 μm²) (Lawson 2002). For each antibody/marker, neurons were considered as positive on the basis of percentage values of neurons that were blindly classed subjectively as clearly stained above background. A subjective score of 1 (visible staining) corresponded to objective AT1R/AT2R values of ≥20% above background which were therefore classed as positive.

**Neurite length measurement in DRG cell culture**

40X images were captured blindly to avoid biased data acquisition. For image analysis, we drew lines over all visible neurites labeled with β-TubIII and measured their length. Then, for each image, we calculated the neurite length per neuron.

**Quantification of nerve terminals in hindpaw skin sections**

10-12 20X randomized images covering most of the section in the epidermal region were captured blindly. Nerve fibers were identified by staining for β-TubIII. CGRP labels peptidergic (likely trkA+) C- and A-fiber neurons, whereas IB4 binds primarily to small unmyelinated C-nociceptor fibers. Due to the fact that IB4 also stains endothelial cells and other non-neuronal cell types (Laitinen 1987), we only quantified as IB4 + nerve terminals those that were also strongly stained for β-TubIII. All visible nerve terminals labeled with β-TubIII/IB4 or β-TubIII/CGRP were sketched and their lengths measured with HCImage. We also measured the whole area encompassing the epidermis and the dermis, and this value was used to calculate neurite length per area.

**Thickness of the epidermis**

We measured the thickness of the epidermis (without stratum corneum) in the contralateral and ipsilateral hindpaw of CFA1 rats with or without AT1R and AT2R antagonists (see above). This was done in images taken from three serial sections (10 sections in between) of the hindpaw skin per rat, at 20X magnification. The thickness of the dermis was measured using a straight line extending from the basal epithelium of the epidermis to the beginning of the stratum corneum in 10 locations per section (Leiguarda et al. 2018).
Hindpaw dorsoventral thickness measurement

Hindpaw dorsoventral thickness was measured by positioning a caliper touching both the ventral and dorsal surfaces of the hindpaw. Care was taken not to compress the hindpaw during measurement.

Statistics

Results are shown as means ± SEM. The n values are provided in the figure legends. The normality of data was assessed with the D’Agostino-Pearson test. For datasets that failed the normality
**Fig. 2** Phenotypic characterization of normal L5 dorsal root ganglion (DRG) neurons expressing angiotensin-II type-2 receptor (AT2R) and their correlation with angiotensin-II type-1 receptor (AT1R). (a and b) Representative photomicrographs of avidin-biotin complex/di-aminobenzidine (ABC/DAB) staining of AT2R and AT1R. Black arrows: AT2R+ neurons. Asterisks: AT2R- large neurons. Plots of either AT2R (n = 117 neurons) or AT1R (n = 223 neurons) cytoplasmic %intensities versus cross-sectional areas (in µm²). Spearman’s correlation values (r_s) and level of significance are indicated in each plot. Horizontal dotted lines mark the limit between negatively stained (intensity < 20%) and positively stained neurons (≥ 20%). (c) Co-localization of AT2R and AT1R. AT2R+/AT1R+ neurons are indicated by white arrows. The bar graph shows the proportions of AT2R+ neurons that are also AT1R- and vice versa. (d) Triple immunofluorescence for AT2R, IB4-binding and trkA. There are AT2R+ neurons positively stained against trkA, IB4 or both phenotypic markers. (e) Plots show that cytoplasmic %intensities of AT2R significantly and positively correlate with IB4 but not with trkA (n = 118 neurons). In the trkA plot, dotted lines indicate three neuronal groups: negatively stained neurons (<20%), weakly trkA+ neurons (20–40% intensity) and strongly trkA+ (>40%). (f) Triple immunofluorescence confocal photomicrographs stained against AT2R, RT97 and trkA. The white arrow points to a possible Aδ-nociceptor. Significance is ***p < 0.001.

**Fig. 3** Central and peripheral projections of AT2R+ peptidergic and non-peptidergic dorsal root ganglion (DRG) neurons. Double immunofluorescence for AT2R (red) and IB4-binding (green) in normal spinal cord (a), dorsal root (b), spinal nerve (c), sciatic nerve (d) and skin (e). There was a modest overlap in the lamina Iii inner (LIIi) between AT2R and IB4. White arrows indicate co-localizations of AT2R with IB4-stained fibers. Arrowheads point to AT2R+ and IB4-fibers. Asterisk in E corresponds to a nerve bundle. (f–h) Double immunofluorescence against AT2R (red) and Substance P (SP, green) in: spinal cord (f), dorsal root (g) and spinal nerve (h). There was a significant overlap in the lamina I (LI) and II outer (LIIo) between AT2R and SP. Most AT2R+ fibers were also stained for SP, as indicated by white arrows. Arrowheads indicate AT2R+ and SP-fibers.
test, correlations were evaluated using the non-parametric Spearman’s test (Spearman’s correlation coefficient, \( r_s \), given when significant). Comparisons between multiple treatment groups were performed with Kruskal–Wallis test plus Dunn’s multiple comparisons test (Figs 5d and 6a,b, and Figure S3) or with one way ANOVA plus Tukey’s multiple comparisons test (Fig. 7g and Figure S4). Comparisons between two treatments groups were evaluated using Mann–Whitney U test (Figs 4a,b, 8a–c, and Figure S5). All tests were performed with Prism 7 (GraphPad software, RRID:SCR_000306). No sample calculation was performed. We did not conduct any data test for outliers and thus no single data points were excluded. Tests were two-tailed and a level of \( p < 0.05 \) was considered statistically significant. Significance is indicated on all graphs by *\( p < 0.05 \), **\( p < 0.01 \), and ***\( p < 0.001 \).

**Results**

**Phenotype of DRG neurons expressing AT2R and AT1R**

Small and medium size neurons are strongly stained for AT2R (black arrows, Fig. 2a). Large neurons (asterisks) show little or no staining. Satellite cells show some weak staining. Quantitative image analysis of 251 neurons from L5 mid-sections (\( n = 5 \) rats) showed that AT2R cytoplasmic intensity was significantly and negatively correlated with cell area (Spearman’s correlation \( r_s = -0.61, p < 0.0001 \), Fig. 2a). AT2R mean ± SEM percentages of cytoplasmic staining were similar for small and medium DRG 61.0 ± 2.2% vs. 54.1 ± 3.1%, respectively, with significantly lower levels in large neurons (6.9 ± 1.0%, \( p < 0.001 \)).

A similar analysis was conducted for AT1R expression in normal L5 DRG. Although sensory neurons of all sizes expressed AT1R, we observed a negative and statistically significant correlation between AT1R cytoplasmic intensities and cell area (\( r_s = -0.49, p < 0.0001 \), Fig. 2b). The mean (±SEM) percentage levels of cytoplasmic AT1R differed significantly for small (61.2 ± 3.1%), medium (46.5 ± 2.6%), and large (29.2 ± 3.0%, \( p < 0.001 \)) neurons.

We next examined the co-expression of AT2R and AT1R, as this is of fundamental importance to assess the role of Ang-II on these neurons. White arrows in Fig. 2c indicate AT2R+ neurons that are also AT1R+. Most AT2R+ neurons were also AT1R+ (188 of 194, 97%), whereas about 80% (188 out of 234) of the AT1R + neurons were also AT2R+. Thus, the majority of angiotensin-sensitive neurons in the DRG express both receptors.

We next characterized the sensory neuron subpopulation that expressed AT2R. Fig. 2d shows the typical pattern of expression for AT2R, binding of IB4 and trkA. As we reported previously (Benitez et al., 2017) most AT2R+ neurons bind IB4, albeit there are some small- and medium-sized DRG neurons who exhibit co-localization of AT2R and trkA. Analysis reveals a positive and significant correlation between AT2R and IB4 (\( r_s = 0.49, p < 0.0001 \)), but no significant correlation with trkA (Fig. 2e).

We occasionally observed punctate, Golgi-like, low intensity AT2R staining in a few large neurons. To determine which type of myelinated A-neurons they were, we examined the co-localization of AT2R with a marker for myelinated DRG neurons (RT-97) and trkA (as a label for A-nociceptors (Fang et al. 2005a)). We found few neurons that evinced at once tenuous AT2R labeling, were trkA + and had visible RT97 (white arrow in Fig. 2f).

**Projections of AT2R+ sensory neurons**

We found AT2R staining mostly in laminae I and II of the dorsal horn of the spinal cord, where it co-localizes partially with IB4 (Fig. 3a), and remarkably well with substance P (Fig. 3f). We also observed substantial axonal co-localization of AT2R with IB4 and SP in the L5 dorsal root as well as in the L5 spinal nerve (indicated by white arrows in Fig. 3b,c,g, h). In both cases, some fibers (indicated by arrowheads) were AT2R+ but negative for either IB4 (Fig. 3b,c) or SP (Fig. 3g, h). As expected, AT2R staining co-localized well within a subset of IB4 + fibers in the sciatic and especially in the sural division of the sciatic nerve (indicated with an arrow, Fig. 3d). Finally, free terminals of C-fiber IB4 + neurons in the skin (white arrows) and nerve bundles (asterisk) were AT2R+ (Fig. 3e).

**Inflammatory soup up-regulates AT2R but not AT1R in vitro**

Selective blockade of AT2R with EMA401 reverted mechanical allodynia associated with cutaneous inflammation (Chakrabarty et al. 2013; Anand et al. 2015). However, the mechanism underlying this effect is poorly understood and so is the possible involvement of AT1R.

Real-time semi-quantitative PCR analysis of three separate DRG cultures showed that addition of an IS for 24 h caused a significant elevation of AT2R mRNA for all three conditions (NTF vs. NTF + IS \( p = 0.0014 \); NGF vs. NGF + IS \( p = 0.0018 \), and GDNF vs. GDNF + IS \( p = 0.0315 \) (Fig. 4a). We noticed that GDNF alone also caused a significant increase in AT2R mRNA (\( p = 0.0121 \)). In contrast, AT1R mRNA levels remained unchanged either by the addition of trophic factors or IS (Fig. 4b). This result does not preclude post-transcriptional regulation that could result in altered protein expression, particularly for AT1R. Furthermore, the analysis by PCR does not discriminate between neuronal subpopulations. Thus, it is important to analyze AT1 and AT2 receptor expressions in vivo, where the neurons are exposed in a more physiological manner to the inflammatory environment.

**CFA cutaneous-induced inflammation regulates AT2R expression**

We next examined whether 1- and 4-day CFA-induced cutaneous inflammation altered the expression of AT2R in primary sensory neurons. We measured AT2R staining intensity at three locations within each neuron: whole...
cytoplasm (excluding the nucleus), at the cell edge and at the perinuclear region (called inner). Large neurons exhibited very low and invariant levels of AT2R (see Figure S3). The whole dataset, including the measurements done in single neurons, all neuronal sizes and conditions for AT1R and AT2R, plus statistical analyses is presented in Tables S1 through S4. Note that the bar plots in Figs 5 and 6 show the average %intensity per rat, but the intensity measurements were carried out neuron by neuron.

Representative photomicrographs (Fig. 5a) and detailed quantification (Fig. 5b) revealed that overall AT2R cytoplasmic intensity was unchanged in small and medium size neurons. However, small neurons showed a significant bilateral increment at the cell edge (55.9 ± 2.4% for contralateral and 57.2 ± 1.3% for ipsi) compared to normal (44.6 ± 2.7%, p = 0.0052). Mean AT2R intensity also increased significantly ipsilaterally relative to normal and contralateral at the inner region of small neurons (69.6 ± 1.4 vs. 61.5 ± 1.9 vs. 61.2 ± 2.6, p = 0.0057). This change in AT2R expression was restricted to small neurons, likely unmyelinated C-neurons.

At CFA4, unlike CFA1, we found a different AT2R expression pattern. First, there was no significant change at any subcellular location in small neurons (Fig. 5c,d). Remarkably, medium size neurons showed statistically significant ipsilateral increments in the average %intensity of AT2R compared to both normal and contralateral at the whole cytoplasm (65.5 ± 1.1 for ipsi vs. 53.6 ± 3.3 for normal vs. 50.2 ± 3.0 for contralateral, p = 0.0021) and at the cell edge (59.8 ± 1.7 for ipsi vs. 37.3 ± 2.3 for normal vs. 46.1 ± 2.2, for contralateral, p < 0.0001). At the perinuclear region (inner) we also observed a significant increment in AT2R mean intensity relative to contralateral (66.4 ± 1.7 vs. 53.8 ± 3.0, p = 0.0081) (Fig. 5d). In summary, our results strongly indicate that AT2R expression increased in small neurons at CFA1 and in medium DRG neurons at CFA4.

CFA cutaneous-induced inflammation regulates AT1R expression

We next studied how cutaneous inflammation affected AT1R expression as its involvement in the peripheral response to inflammation remains unclear. Fig. 6 summarizes our findings on the expression pattern of AT1R in small, medium, and large DRG neurons at CFA1 (A) and CFA4 (B). We observed no changes whatsoever in AT1R mean intensity at CFA1 either at the cytoplasm, the cell edge or inner for any neuronal subpopulation, except for a modest increment in whole cytoplasm level contralaterally in large neurons (Fig. 6a). Interestingly, at CFA4 we found elevated ipsilateral average %intensity of AT1R in large neurons with no changes in small or medium subpopulations. This increment was statistically significant for ipsilateral vs. normal and contralateral DRG in the cytoplasm (42.7 ± 1.7
vs. 29.6 ± 1.3 for normal and 31.3 ± 2.4 for contralateral, p = 0.004). Similarly, AT1R levels increased significantly at the cell edge (46.1 ± 2.2 for ipsi vs. 34.5 ± 2.1 for normal and 34.2 ± 2.0 for contralateral, p = 0.0055). Finally, we found elevated inner AT1R average intensity for ipsilateral compared to normal (42.5 ± 2.8 vs. 29.3 ± 1.2, p = 0.0007). Thus, in contrast to the case for AT2R, AT1R expression changed significantly only in large neurons and at CFA4.

**In vitro neuritogenesis of DRG neurons depends on AT1R and AT2R**

The extent of skin innervation has been linked to pain sensitivity with inflammation leading to an increase in
Fig. 7 Angiotensin-II type-1 receptor (AT1R) and angiotensin-II type-2 receptor (AT2R) contributed to the increase in neurite length induced by Ang-II in dorsal root ganglion (DRG) neurons in vitro. Representative confocal micrographs of all six experimental conditions are shown: no trophic factors (a), nerve growth factor (NGF) (b), Ang-II (c), Ang-II + PD123319 (d), Ang-II + Azilsartan (e), and Ang-II + PD123319+Azilsartan (f). The far right column shows the masks generated to measure the total neurite length in each experimental condition. (g) Bar plot shows the neurite length (µm) per neuron in all conditions. Ang-II promoted neurite outgrowth in these neurons, an effect that was reverted by blocking AT1R with azilsartan and also by inhibiting AT2R with PD123319. Each dot represents the average neurite length measured in the neurons of 1 coverslip. Data from three separate cultures from three independent rats. Significance is as follows *p < 0.05 and **p < 0.001.

neurite outgrowth. It is also known that inflammation triggers the release of Ang-II and that this molecule induces neuritogenesis in several neuronal types, including primary sensory neurons (Chakrabarty et al. 2008; Anand et al. 2013; Chakrabarty et al. 2013). In light of our observations regarding the expression of AT1R and AT2R after CFA-induced inflammation, we decided to evaluate the roles of these two receptors under controlled conditions with cultured DRG neurons.

To this end, we tested the effect of Ang-II on neurite outgrowth (see Methods). We used NGF 7S as a positive control due to its strong neurite outgrowth promoting activity (Delree et al. 1989). Figure 7(a–f) shows representative confocal micrographs of all experimental conditions with their staining against β-TubIII, AT2R and AT1R plus the mask generated to measure total neurite length in each condition. We stained against AT1R and AT2R to demonstrate that in vitro DRG neurons maintain the expression of the receptors and also to exclude the possibility that a lack of effects of Ang-II was due to receptors down-regulation. We did not observe any obvious change in the expression of either AT1R or AT2R. The bar plot in Fig. 7g shows the neurite length per neuron under all conditions. As expected NGF had a strong neurite-promoting activity (411.1 ± 48.7 µm/neuron) compared to NTF (165.5 ± 20.5 µm/neuron, p < 0.0001). Ang-II also exhibited statistically significant neuritogenic activity compared to NTF (307.3 ± 27.5 µm/neuron, p < 0.0043) and was not significantly different from NGF. Interestingly, treatment with the AT2R antagonist PD123319 (Ang-II + PD) and azilsartan (a selective competitive inhibitor of AT1R; Ang-II + Azil) resulted in significantly lower average neurite length than with Ang-II alone (154.9 ± 41.8 µm/neuron for PD and 174.9 ± 25.2 µm/neuron p < 0.003 for Azil). This suggests that Ang-II exerted its neuritogenic action via both AT1R and AT2R. Thus, it seems that the individual inhibitors completely blocked the activity of Ang-II and the combination was below NTF control, although it is not statistically different (p = 0.058). Note that we obtained the same pattern of neurite outgrowth in the different experimental conditions when measuring neurite length using PGP9.5 instead of β-TubIII (Figure S4), to control for a possible artifact arising from neurite thickness-related staining intensity.

In vivo pharmacological manipulation of AT1R and AT2R affects peripheral neurite density

To determine whether our findings in vitro were physiologically meaningful, we looked for an Ang-II effect on neuritogenesis in vivo. Of note, one study has already established that antagonizing AT2R with PD123319 affects CFA-induced neuritogenesis (Chakrabarty et al. 2013). However, the role of AT1R in this process was not examined. Thus, we evaluated whether selective in vivo pharmacological inhibition of AT1R and both AT1R and AT2R altered a) the inflammatory response and b) the neurite density of IB4 + and CGRP + terminals.

Figure S5A shows the induction of edema at CFA1 in the ipsilateral (CI) vs. contralateral (CC) hindpaw. Losartan failed to reduce or prevent the formation of this edema (Los I vs. Los C) and so did the combination of 4 weeks losartan plus 3 days PD123319 (Los + PD 1 vs. Los + PD C). Another marker of inflammation is the thickness of the epidermal layer, which increases after CFA treatment (Leiguarda et al. 2018). At CFA1, we found that the mean epidermal thickness increased ipsilaterally under all experimental conditions (Figure S5B). This confirms our finding that neither chronic low dose losartan nor the combination of losartan plus PD at the concentrations we used was effective in reducing or preventing edema. We also observed thickened epidermal layer in the contralateral side of losartan-treated rats compared to control. This may be due to the ability of losartan to induce significant elevation in circulating levels of the growth factors receptors EGFR and KGFR (Koga et al. 2008).

Because previous reports showed apparent no change in neurite outgrowth with CFA but lacked proper quantification (Chakrabarty et al. 2013) we studied the effect of Los and Los + PD on the neurite density of myelinated terminals. We found that the density of myelinated terminals increased significantly after CFA in control rats (p = 0.0151). This increment was not prevented by Los (p = 0.0041) but was suppressed by Los + PD (Fig. 8a).

We also examined the impact of the treatments in IB4 + free terminals (Fig. 8b). As expected, control rats exhibited a marked increment in the density of IB4 + neurites ipsilaterally at CFA1 (p = 0.017). Rats treated with Losartan showed no ipsilateral increment in neurite density compared to the contralateral side. These rats also exhibited a
Fig. 8 In vivo angiotensin-II type-1 receptor (AT1R) and angiotensin-II type-2 receptor (AT2R) antagonists altered the CFA-induced increment in neurite density of the hindpaw skin. Representative photomicrographs of ipsilateral skin of the hindpaw with (a) RT97 and 4',6'-diamidino-2-phenylindole (DAPI); (b) IB4-binding and β-TubIII; (c) calcitonin-gene-related peptide (CGRP) and β-TubIII. Three experimental groups are shown: CFA1, CFA1 in rats pre-treated with oral losartan (CFA + Los) and CFA1 in rats pre-treated with oral losartan plus i.p. PD123319 for 3 days prior to CFA injection (CFA + Los+PD). Bar plots show the density of the visible nerve terminals for each marker in the contralateral and ipsilateral paw in the three experimental groups (n = 6 rats per group). White arrows point to nerve terminals that were quantified. Dotted lines demarcate the limit between the epidermis (Epi) and the endodermis. Significance is as follows *p < 0.05 and **p < 0.01.
much lower level of neuritogenesis than control rats (CI vs. Los I, \( p = 0.007 \)). Although there was no statistically significant reduction in IB4 + neurite density contralaterally, the trend was for these animals to exhibit less neuritogenesis than control rats. Surprisingly, the co-administration of PD123319 with Los resulted in a drastic reduction in IB4 + neurite density contralaterally compared to control (\( p = 0.003 \)). This treatment also resulted in very low levels of innervation ipsilaterally, although CFA still caused a significant increment when compared with the contralateral foot. Our findings indicate that simultaneously blocking AT1 and AT2 receptors has a systemic, bilateral arresting effect on neuritogenesis of IB4 + C-neurons.

Finally, we found that Losartan treatment abolished the ipsilateral up-regulation of peptidergic fibers neuritogenesis induced by CFA (there was no difference between Los C vs. Los I). The addition of PD did not change this effect (\( p = 0.0519 \)) (Fig. 8c). Therefore, it seems that in the case of peptidergic terminals signaling of Ang-II through AT1R is dominant over AT2R.

**Discussion**

Here we show a full phenotypic characterization of primary sensory neurons that express AT2R. We also demonstrate a unique change in the pattern of expression of AT1R and AT2R in different neuronal subpopulations at different times in response to cutaneous inflammation. We provide the first demonstration of a direct regulation of AT2R *in vitro* by an inflammatory soup and also evidence that GDNF could up-regulate this receptor. We establish the co-localization of AT1R and AT2R and examined in detail their interplay as mediators of Ang-II induced neurite outgrowth *in vitro*. We found selective effects of Ang-II on *in vivo* neuritogenesis of non-peptidergic C-fiber neurons, CGRP + nerve terminals and also myelinated fibers by pharmacologically manipulating AT1R and AT2R. Taken together, our data indicate that in the DRG neurons exposed to inflammation, AT1R and AT2R act co-operatively instead of antagonizing each other, as it is the canonical view.

Our phenotypic multifaceted characterization using antibodies and pharmacological tools confirms and expands previous published results showing the presence of AT2R at sites where its activation could lead to the regulation of nociception (Anand *et al.* 2013; Chakraborty *et al.* 2013; Benitez *et al.* 2017). Note that the use of commercially available antibodies has some limitations (Goodman 2018). The specificity of the antibodies against AT2R and AT1R has been questioned (Hafko *et al.* 2013). Thus, we carried out a validation of the antibodies used in this study (Figure S1). Nonetheless, we acknowledge that the results coming from their use must be interpreted with caution.

Our data show that AT2R is preferentially expressed in non-peptidergic (IB4-binding) likely C-nociceptors in line with our previous study (Benitez *et al.* 2017). The receptor was also present in peptidergic (trkA+/SP+) neurons that encompass both small C-nociceptors and also medium size A-neurons (Fang *et al.* 2005a). The latter include the type II A-mechanoheat units with moderate conduction velocities and higher mechanical and lower heat thresholds (Djouhri and Lawson 2004). Low levels of AT2R in a few trkA+/RT97 + large neurons may indicate that the receptor was also present in Aβ-nociceptors. These neurons are high-threshold mechanoreceptors thought to represent ~ 20% of all Aβ/δ neurons in the DRG (Fang *et al.* 2005b; Dubin and Patapoutian 2010).

We found that AT1R in all neuronal sizes, although its expression was highest in small neurons, in agreement with previous studies (Pavel *et al.* 2008; Patil *et al.* 2010). The fact that AT1R and AT2R are often present in the same neurons warranted examination of their possible interplay in the context of cutaneous inflammation. Our first observation showed that exposure of purified DRG cultures to a complex inflammatory soup for 24 h caused an increment in the mRNA levels of AT2R but not of AT1R. This suggested that a similar mechanism might be operating during chronic cutaneous inflammation.

It is interesting to note that GDNF and its high-affinity receptor GDFRx1/Ret in IB4 + neurons are involved in hyperalgiesic priming (Joseph and Levine 2010) and also in persistent muscle pain (Alvarez *et al.* 2012). We found that GDNF stimulates AT2R mRNA synthesis in DRG cultures, an observation that agrees with the known GDNF-dependence of survival and phenotypic differentiation of IB4-binding neurons (Zwick *et al.* 2002).

Our finding that CFA1 increased AT2R expression in small, C-like neurons, whereas CFA4 increased the receptor expression in medium A-neurons indicates that AT2R is selectively and differentially modulated throughout the inflammation process. To our knowledge, this is the first time this unique pattern has been reported. Our observed simultaneous increment in AT2R at the inner and edge probably indicates enhanced synthesis *de novo* plus transport toward the membrane. This aspect of the change in expression requires further examination but agrees with studies showing that enhanced membrane trafficking of pain-facilitating molecules underlies the response to inflammation and nerve damage [reviewed by (Ma and Quirion 2014)].

In contrast to the pattern seen with AT2R, AT1R expression remained largely unchanged in all cell sizes and subcellular locations at CFA1 but increased significantly in large neurons at CFA4. To put this surprising finding in context, other researchers found that in a model of CCI the administration of Ang-II led to an increase in the number of large ATF3 + neurons (a marker of neuronal damage). This effect was reverted by treatment with losartan but not with PD123319 (Pavel *et al.* 2013). Also, there is evidence that selective activation of non-nociceptive Aβ neurons can
induce neuropathic pain (Tashima et al. 2018). Thus, there is a possible functional link between likely Aβ/β low-threshold mechanoreceptors or may be Aβ-nociceptors and AT1R, but the underlying mechanism and its physiological meaning remains unclear. One possible interpretation for this unique pattern of change in the expressions of AT1R and AT2R is that AT2R predominantly expressed in small likely C- nociceptive neurons is initially recruited by the inflammation, producing primary mechanical and heat hyperalgesia as part of the nociceptive response to the insult. At 4 days after CFA, the inflammation can then recruit large neurons carrying AT1R. This second phase is associated with the adaptive response to inflammatory pain, and often involves A-fiber like non-nociceptive neurons.

Chronic inflammation is associated with pathological pain. One mechanism proposed to underlie this connection is that pro-inflammatory mediators promote neuritogenesis of both peptidergic and non-peptidergic nociceptors and possibly their sensitization (Anand et al. 2013). This hyper-innervation has been shown in skin [reviewed in (Hendrix and Peters 2007)] and in a model of vestibulodynia (Chakrabarty et al. 2018). Thus, we decided to examine the impact of Ang-II on DRG neurite outgrowth, first \textit{in vitro} and then \textit{in vivo}. In our \textit{in vitro} assays to examine neuritogenesis we found that both AT2R and AT1R stimulates neurite outgrowth. This agrees with a previous report that Ang-II enhanced neurite outgrowth in cultured DRG neurons, an effect that was prevented by treatment with an AT2R antagonist (Anand et al. 2013). A similar role for this receptor was seen in CFA-induced vestibulodynia where disruption of the RAS system and antagonism of AT2R attenuated neuritogenesis (Chakrabarty et al. 2018; Sharma et al. 2018). Thus, our observation that in vivo chronic losartan failed to impair the inflammation-induced increment in myelinated terminals while adding the AT2R blockade succeeded, suggests that there was a subpopulation of large AT1R + neurons whose neuritogenesis was independent of Ang-II. However, the fact that blocking both receptors prevented CFA-induced neurite growth of these neurons suggests the existence of a paracrine control mechanism involving AT2R. We speculate that an unknown factor released by AT2R+ neurons (different from Ang-II) may be controlling the neuritogenesis in these large myelinated neurons. An alternative interpretation is that the subpopulations that respond to inflammation are the A and Aβ-nociceptors which would be affected by dual receptor blockade but not by AT1R blockade alone, as the neurite outgrowth in large neurons appears to be Ang-II independent.

Here we provide the first demonstration that IB4 + C-fiber neuritogenesis depends (at least partially) on recruiting AT1R and AT2R upon CFA-induced release of Ang-II. As IB4 + neurons mediate mechanical and heat hyperalgesia in the rat (Tarpley et al. 2004; Cavanaugh et al. 2009) any significant alteration in their nerve endings (length, reach and branching) might affect their ability to respond to noxious stimuli. This agrees well with previous reports indicating that antagonizing AT2R with EMA401 reduced mechanical allodynia in a rat model of CFA (Anand et al. 2015) and also proved efficacious in the treatment of post-herpetic neuralgia in a limited clinical trial (Rice et al. 2014). However, no previous studies have associated AT1R with neurite outgrowth of IB4 + neurons. One report showed that blockade of the AT1 receptor with losartan in a rat model of CCI prevented tactile hyperalgesia and attenuated cold hyperalgesia, but failed to affect the response to noxious heat stimulus (Pavel et al. 2013). Thus, we propose that a reduction in the neurite length of IB4 + neurons may underlie this effect. One unexpected result was the significant reduction in contralateral IB4 + neurites with the dual blockade. This might result from a more substantial systemic...
effect on the contralateral side, whereas other neurite-promoting molecules (RAS-independent) are active ipsilaterally.

Regarding peptidergic nerve endings (these include C, A, and some Aβ trkA + nociceptors) we found that AT1R blockade abolished the ipsilateral up-regulation of neuritogenesis induced by CFA. Surprisingly, the addition of AT2R blockade did not modify this effect. Note that treatment with PD123319 alone in CFA-treated rats showed that AT2R is involved in CGRP-like long-term hyperinnervation of the skin (Chakrabarty et al. 2013). However, unlike our study, these authors did not examine either the effect of blocking AT1R alone or both receptors. Thus, it is possible that the discrepancy between our findings and theirs is due to the interplay between the receptors.

Additionally, as we performed the in vitro studies in P15 DRG neurons and the in vivo pharmacology in P90 rats, discrepancy in the results might be expected reflecting age-related differences in physiology. However, what we observed in vitro confirms what we found in vivo.

Accumulating evidence suggests that regulation of mutually antagonistic AT1R and AT2R is essential for maintaining control of inflammation and that an imbalance between these two receptors is potentially pathological (Smith and Missailidis 2004). Most papers in the literature have focused on either AT1R or AT2R but it is important to remember that any C- or A-fiber neuron exposed to Ang-II will have both receptors activated simultaneously. Depending on the relative levels of the two receptors (variable among neuronal subpopulations and pathologies) and how they affect each other the net outcome of their activation can be very different. Thus, the key may lie in the balance between the level of expression and activity of the two receptors. Using an experimental paradigm similar to ours, (Hashikawa-Hobara et al. 2012) showed that administration of the AT1R blocker candesartan improved Ang-II pro-neuritogenic activity mediated via AT2R.

In conclusion, ARBs have recently been promoted as potential drugs to alleviate chronic pathological pain associated with nerve injury and inflammation. However, most studies have underplayed the potential cross-talk between AT1R and AT2R. This would be particularly relevant for patients using anti-hypertensive regimes based on ARBs (mostly AT1R antagonists) who rely on AT2R antagonists as a palliative for pain. Given the range of neuronal subpopulations expressing AT1R and AT2R, their co-localization in these neurons and their significant interplay, it might be beneficial to examine mecanophoreception and proprioception and the presence of paresthesia/dysesthesia in these patients.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Author contributions

SB, AS, DM, SP, and CA designed experiments, analyzed data, and wrote the manuscript. SB, DM, MF, and CA performed the experiments. AS and SP provided essential reagents and promoted the study. AS and CA conceived and managed the study.

Compliance with ethical standards

Animals used in this study were cared for in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee (CICUAL 31/2014 and 102/2017) of the Universidad Nacional de Cuyo, Mendoza, Argentina.

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Supporting information

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**Figure S1.** Characterization of anti-AT1R and AT2R antibodies. **Figure S2.** Measurement of staining intensity. **Figure S3.** Large DRG neurons express very low levels of AT2R that do not change at CFA1 or CFA4. **Figure S4.** AT1R and AT2R contributed to the increase in neurite length induced by Ang-II in DRG neurons in vitro. **Figure S5.** Effect of chronic oral losartan alone or combined with PD123319 on foot and epidermis thickness. **Table S1.** Summary of %intensities of AT2R measured in mid sections of L5 DRG from normal rats and 1 day after CFA-induced cutaneous inflammation.(contralateral and ipsilateral). **Table S2.** Summary of %intensities of AT2R measured in mid sections of L5 DRG from normal rats and 4 days after CFA-induced cutaneous inflammation (contralateral and ipsilateral).
Table S3. Summary of %intensities of AT1R measured in mid sections of L5 DRG from normal rats and 1 day after CFA-induced cutaneous inflammation (contralateral and ipsilateral).

Table S4. Summary of %intensities of AT1R measured in mid sections of L5 DRG from normal rats and 4 days after CFA-induced cutaneous inflammation (contralateral and ipsilateral).

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