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A Small Molecule Angiotensin II Type 2 Receptor (AT$_2$R) Antagonist Produces Analgesia in a Rat Model of Neuropathic Pain by Inhibition of p38 Mitogen-Activated Protein Kinase (MAPK) and p44/p42 MAPK Activation in the Dorsal Root Ganglia

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Disclosures

Abstract

Objective. There is an unmet clinical need for novel analgesics for neuropathic pain. This study was designed to elucidate the mechanism through which EMA300, a small molecule antagonist of the angiotensin II type 2 receptor (AT$_2$R) with >1,000-fold selectivity over the angiotensin II type 1 receptor, produces analgesia in a rodent model of neuropathic pain.

Design and Methods. Groups of AT$_2$R knockout, hemizygotes, and wild-type mice with a chronic constriction injury (CCI) of the sciatic nerve received single intraperitoneal (i.p.) bolus doses of EMA300 (100 or 300 mg/kg), and analgesic efficacy was assessed. Groups of control, sham-operated, and CCI rats were euthanized and perfusion fixed. Lumbar dorsal root ganglia (DRGs) were removed for investigation of the mechanism through which EMA300 alleviates neuropathic pain.

Results. EMA300 analgesia was abolished in AT$_2$R knockout CCI mice with intermediate responses in the hemizygotes, affirming the AT$_2$R as the target mediating EMA300 analgesia. In CCI rats, DRG immunofluorescence (IF) levels for angiotensin II, the main endogenous ligand of the AT$_2$R, were increased ~1.5–2.0-fold ($P < 0.05$) cf. sham-controls. Mean DRG IF levels for activated p38 (pp38) and activated p44/p42 (pp44/pp42) MAPK were also increased ~1.5–2.0-fold ($P < 0.05$) cf. sham-controls. At the time of peak EMA300 analgesia in CCI rats, mean DRG levels of pp38 MAPK and pp44/pp42 MAPK (but not angiotensin II) were reduced to match the respective levels in sham-controls.

Conclusion. Augmented angiotensin II/AT$_2$R signaling in the DRGs of CCI rats is attenuated by EMA300 to block p38 MAPK and p44/p42 MAPK activation, a mechanism with clinical validity for alleviating neuropathic pain.

Key Words. Angiotensin II Type 2 Receptor (AT₂R); Neuropathic Pain; Analgesia; p38 Mitogen Activated Protein Kinase (MAPK); p44/p42 MAPK; Angiotensin II; Dorsal Root Ganglia

Introduction

There is a large unmet clinical need for novel analgesics with improved efficacy and tolerability profiles for the treatment of neuropathic pain [1]. Hallmark features of neuropathic pain include hyperexcitability of primary afferent nerve fibers and abnormal sprouting of dorsal root ganglion (DRG) neurons [2]. Hence, drug discovery strategies aimed at inhibiting the mechanisms underpinning these features may identify molecules with potential as novel analgesics for improved relief of neuropathic pain.

Our laboratory has recently shown that several small molecule antagonists of the angiotensin II type 2 receptor (AT_2R) with >1,000-fold selectivity over the angiotensin II type 1 receptor (AT_1R), produced dose-dependent analgesia in rats with a chronic constriction injury (CCI) of the sciatic nerve, a widely utilized rat model of neuropathic pain [3]. Hence, the present study was designed to investigate the mode of action through which AT_2R antagonists produce analgesia in neuropathic pain.

The AT_2R is expressed in the brain and viscera of adult mice [4] and on small- to medium-sized DRG neurons in the adult rat and humans [5]. In human tissue sections, the AT_2R is expressed on nerve fibers in peripheral nerves, skin, urinary bladder, and bowel [5]. In cultured neuronal cells, angiotensin II, the main endogenous ligand of the AT_2R, induces neuronal excitability [5,6] and neurite outgrowth [5,7], with these effects blocked by the selective small molecule AT_2R antagonists, PD123319 [7] and EMA401 [5].

Angiotensin II signaling via the AT_2R does not modulate blood pressure *in vivo* and its physiological role remains enigmatic (see Reference [8] for review). In terms of safety evaluation in healthy human subjects, brief (5 minutes) intravenous infusion of PD123319 (also known as EMA200) that has >1,000-fold selectivity over the AT_1 receptor [3], at 10 µg/kg or placebo in a randomized, double-blind, crossover clinical study in 16 healthy human subjects was well-tolerated [9]. In particular, PD123319 did not significantly alter measures of cardiovascular function, including blood pressure, mean arterial pressure, cardiac index, systemic vascular resistance index, reflective index, and arterial stiffness index [9]. For the orally active small molecule AT_2R antagonist, EMA401, that has 10,000-fold specificity over the AT_1 receptor [3], a 4-week double-blind, randomized, placebo-controlled clinical study in 183 patients with postherpetic neuralgia showed that it produced significant analgesia above placebo and was well-tolerated [10]. EMA401 does not cross the blood–brain–barrier [5], which is consistent with its lack of central nervous system side-effects [10].

It is well-known that capsaicin, the pungent component of hot chili peppers, activates the transient potential vanilloid receptor 1 (TRPV1) in sensory neurons, resulting in calcium influx, neuronal excitability, and pain [11]. In cultured human DRG sensory neurons, capsaicin-induced excitability is augmented by angiotensin II, with these effects attenuated in a concentration-dependent manner by the selective AT_2R antagonist, EMA401 [5]. By contrast, the selective AT1R has already been abbreviated on line 15/16, Page 2 (this page) (AT_1R) antagonist, losartan, had no effect on capsaicin-induced excitability in cultured human DRG sensory neurons [5].

In cultured cells of neuronal origin (NG108-15 cells), angiotensin II/AT_2R-induced neurite outgrowth is underpinned by persistent activation (phosphorylation) of the putative chronic pain marker, p44/p42 mitogen-activated protein kinase (MAPK) [12,13]. MAPK is a key enzyme in the phosphorylation of major voltage-gated ion channels such as $Na_v1.8$ [14] and $Na_v1.7$ sodium channels [15,16], N-type ($Ca_v2.2$) calcium channels [17] and TRPV1 [18], all of which are expressed in DRG neurons and are implicated in nerve injury-induced sensory neuron hyperexcitability and neuropathic pain.

Painful neuromas in humans [19] and experimental neuromas in rats [16] contain high expression levels of activated p38 MAPK and p44/p42 MAPK, and a recent clinical trial showed that the p38 MAPK inhibitor, dilmapimod, produced significant analgesia in patients with neuropathic pain [20,21]. Here, we show for the first time that EMA300-evoked analgesia in CCI mice was abolished by genetic deletion of the AT_2R affirming that the AT_2R is the receptor target for EMA300. Additionally, we found that the analgesic effects of EMA300 in CCI rats are mediated by inhibition of p38 MAPK and p44/p42 MAPK activation in the ipsilateral lumbar DRGs, a mechanism with clinical validity for relief of neuropathic pain in patients [20,21].

Materials and Methods

Animals

Approval was obtained from the Animal Ethics Committee of The University of Queensland for the animal experiments described herein and experiments adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004). Adult male Sprague-Dawley (SD) rats and female C57BL/6J mice were purchased from The University of Queensland Biological Resources. Female AT_2R knockout (KO) mice and male hemizygote mice were obtained from the Baker Heart Research Institute in Melbourne (Australia). These mice were received into the Baker as the original strain (129SV/C57BL6J background from T. Walther, Charitè animal facilities (FEM), Freie Universität Berlin, Germany). They were backcrossed onto C57BL/6 for five or six generations. Genome scanning was performed by Jackson Labs on generation 5 knockout female and generation 6 male hemizygote mice; 97% and 99% C57BL/6 markers were observed, respectively. The mice were shipped to The Transgenic Animal Service of Queensland (The University of Queensland) and the progeny were used for
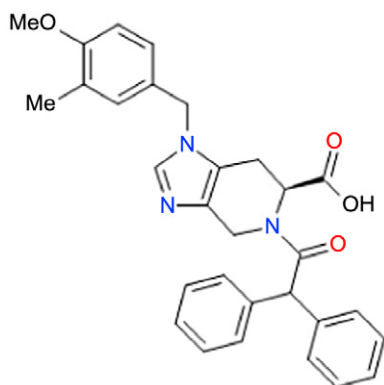
Figure 1 Chemical structure of EMA300 (also known as PD121,981).

these experiments. Tails were tipped, and PCR analysis was used to establish whether mice were homozygote AT_2R KO or heterozygotes. Animals were housed in a temperature-controlled facility (21°C ± 2°C) with a 12 h/12 h light–dark cycle, and experimentation was conducted during the light phase. Standard laboratory rodent chow and water were available *ad libitum*.

Test Compound

The selective AT_2R antagonist EMA300 (also known as PD121,981 as the sodium salt; Figure 1) was synthesized by Glycosyn[irl] (Lower Hutt, New Zealand) and supplied by Spinifex Pharmaceuticals Pty Ltd (Melbourne, Australia).

Induction of a CCI in Rats and Mice

A unilateral CCI of the sciatic nerve was induced in rats and mice according to a published method [22]. Briefly, rodents were anaesthetized with 3% isoflurane delivered in oxygen, and a CCI was induced by tying four loose ligatures (rats) or three loose ligatures (mice) that were ~1 mm apart around one sciatic nerve. Sham-operated animals underwent similar surgery, including the exposure of the sciatic nerve but omitting the tying of loose ligatures. Development of mechanical hypersensitivity in the hind-paws was determined using von Frey filaments to measure paw withdrawal thresholds (PWT values) in the ipsilateral (injured side) hindpaws presurgery and at 14-days post-CCI surgery in rats, as well as at 7- and 14-days post-CCI surgery in mice.

Assessment of Mechanical Allodynia in the Hindpaws

Von Frey filaments (Stoelting) were used to determine the lowest mechanical threshold required to evoke a brisk paw withdrawal reflex in the rat hindpaws. Briefly, rats or mice were transferred to wire mesh testing cages and allowed to acclimatize for 15–20 minutes prior to von Frey testing. Commencing with the 6 g or the 0.6 g filament for the rat or mouse, respectively, the filament was applied to the plantar surface of the hindpaw until the filament buckled slightly. Absence of a response after 3 seconds prompted use of the next filament of increasing force. Conversely, a hindpaw withdrawal response within 3 seconds prompted use of the next filament of decreasing force.

A score of 20 g was given to rats that did not respond to any of the von Frey filaments in the range 2–20 g. Mechanical allodynia in rats was fully developed when von Frey paw withdrawal thresholds (PWTs) in the ipsilateral hindpaws were ≤6 g. The treatment goal is to increase von Frey PWTs in the injured hindpaws from ≤6 g to prenerve injury values (~12 g), which represents complete alleviation of mechanical allodynia.

In mice, a score of 8 g was given to animals that did not respond to any of the von Frey filaments (0.008–8 g). Mechanical allodynia was fully developed when von Frey PWTs in the injured hindpaws were ≤0.8 g. The treatment goal is to increase von Frey PWTs in the hindpaws from ≤0.8 g to prenerve injury values (~1.2–1.6 g), which represents complete alleviation of mechanical allodynia.

Test Compound Administration in Rodents

Animals received single bolus doses of EMA300 (Figure 1) or vehicle by the intraperitoneal (i.p.) route, and PWTs were assessed in the hindpaws using von Frey filaments predose and at the following postdosing times: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, and 3 hours. Vehicle comprised water for injection.

CCI Mice

Groups of drug-naïve wild-type CCI mice received single i.p. bolus doses of EMA300 at 100 mg/kg (N = 8), 300 mg/kg (N = 3) or vehicle (N = 5). These doses of EMA300 were from the middle and the top of the dose–response, curve respectively. Groups of drug-naïve AT_2R knockout CCI mice and hemizygous CCI mice received single i.p. bolus doses of EMA300 at 100 mg/kg (N = 6 per group) and 300 mg/kg (N = 3 per group).

CCI Rats

Drug-naïve CCI rats with fully developed mechanical allodynia, sham-operated and age-matched nonoperated rats (N = 5–6 rats per group) were euthanized at 14 days postsurgery and tissues were collected for immunohistochemical analysis. A separate group of CCI rats (N = 4) was administered the ~ED_{80} dose of EMA300 (10 mg/kg i.p.) at 14 days post-CCI surgery and euthanized at the time of peak analgesia (1 hour postdosing).

Tissue Collection and Preparation

After euthanasia with an overdose of pentobarbitone (Lethabarb® Virbac Australia Pty Ltd, Sydney, Australia), rats (N = 5–6 per group) and mice (N = 4–5 per group) were immediately perfused transcardially with 0.1 M phosphate buffered saline (PBS; 25°C) or saline, respectively.

This was followed by 4% PFA (Proscitech, Thuringowa, QLD, Australia) in 0.1 M PBS, pH 7.4 (4°C) (~200 mL for rats and ~100 mL for mice). The L4-L6 DRGs were collected following laminectomy and the adrenal glands of mice were also removed. Tissues were postfixed for 2–4 hours in this solution (4°C), cryoprotected for 5 days in 30% sucrose/PBS at 4°C, and then placed in a 1:1 mixture of OCT:30% sucrose/PBS at 4°C followed by freeze-mounting in OCT embedding medium (Proscitech) and stored at –80°C until used. Frozen DRG and adrenal gland sections (10–12 μm) were cut using a cryostat, mounted on slides and stored at –20°C until used.

Immunostaining and Morphological Assessment

The comparative specificity of Abcam and Santa Cruz anti-AT_2R antibodies were assessed using sections of the adrenal glands and the lumbar DRGs of female wild-type and AT_2R KO mice. The primary antibodies used were anti-AT_2R (rabbit polyclonal (ab19134), 1:250, Abcam, Cambridge, MA, USA) and antihuman AT_2R receptor (rabbit polyclonal (H-143) or sc-9040, 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The secondary antibody used was Alexa Fluor® 488 goat antirabbit IgG (H + L) (1:5,000, Invitrogen Australia, Melbourne, Australia).

p38, p44/p42, Angiotensin II, Substance P and NF200

Rat DRG tissue sections were air-dried for 30 minutes prior to postfixation in ice-cold acetone for 5 minutes, washed with PBS, and exposed to blocking solution (3% goat serum, 0.1% Triton X, in PBS) for 30 minutes at room temperature. Subsequently, primary antibodies against phospho-p38 (pp38) MAPK (mouse monoclonal; 1:100, Cell Signaling Technology Inc. Beverly, MA, USA), phospho-p44/p42 (pp44/pp42) MAPK (mouse monoclonal; 1:100, Cell Signaling Technology Inc.), angiotensin II (guinea pig polyclonal; 1:500, Bachem, Bubendorf, Switzerland), substance P (mouse monoclonal; 1:20, Abcam, Cambridge, MA, USA) and NF200 (mouse monoclonal; 1:500, Sigma-Aldrich, Sydney, Australia) were added and incubated in a humidified chamber for 24 hours at 4°C. After washing, the appropriate secondary antibody of goat antimouse Alexa Fluor 555 (1:2,000, Invitrogen Australia, Victoria, Australia) or goat antiguinea pig Alexa Fluor 488 (1:600, Invitrogen Australia) was added, and the tissue was incubated at room temperature for 90 minutes. Slides were then washed and mounted using Prolong Gold (Invitrogen Australia).

NeuN, NGF, Trk A, AT_2R, Substance P and NF200

Rat DRG tissue sections were washed with 11X PBS (pH 7.4):0.3% Tween 20 (Sigma-Aldrich) (PBST) solution (2 × 10 minutes) and were preincubated with Image-iT® FX signal enhancer (Invitrogen Australia) for 2 hours at room temperature, followed by pre-incubation with 0.3% hydrogen peroxide (Sigma-Aldrich) in methanol for 30 minutes. Sections were incubated overnight at 4°C with the primary antibodies (diluted in PBST) against the AT_2R

(rabbit polyclonal anti-AT_2R (ab19134), 1:250, Abcam), tyrosine kinase A receptor (goat polyclonal anti-TrkA, 1:10, Sapphire Bioscience Pty Ltd, Sydney, Australia), NGF (sheep polyclonal anti-NGF (ab49205), 1:100, Abcam, Cambridge, MA), neuronal nuclei (NeuN) (mouse monoclonal anti-NeuN antibody, clone A60, 1:100, Millipore, Temecula, CA, USA), substance P (mouse monoclonal antisubstance P [SP-DE4-21] antibody, 1:1,000, Abcam) and NF200 (mouse monoclonal anti-NF200 antibody, 1:100, Abcam). Subsequently, sections were washed (2 × 10 minutes) in PBST and incubated with the secondary antibodies diluted in PBST, for 2 hours at room temperature. Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1:5,000, Invitrogen Australia, Catalog No. A-11008), Alexa Fluor 546 donkey antigoat IgG (H + L) (1:5,000, Invitrogen Australia, Catalog No. A-11056), Alexa Fluor 633 donkey antisheep IgG (H + L) (1:5,000, Invitrogen Australia, Catalog No. A-21100), Alexa Fluor 700 goat antimouse IgG (H + L) (1:5,000, Invitrogen Australia, Catalog No. A21036) and Alexa Fluor 546 goat antimouse IgG (H + L) (1:5,000, Invitrogen Australia, Catalog No. A11003). Sections were then incubated with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol solution for 5 minutes. Sections were washed in PBST and cover-slipped with ProLong® Gold antifade reagent with DAPI (Invitrogen Australia, Catalog No. P36935).

Image Acquisition

Sections were visualized using an Axioskop 40 microscope (Carl Zeiss, Gottingen, Germany). Images were acquired using a Axiocam MRm camera (Carl Zeiss) and analyzed for degree of immunofluorescence (IF) using Axiovision Rel. v4.8 software. There was an absence of fluorescence in negative controls where the primary antibody was omitted.

Data Analysis

Paw Withdrawal Thresholds in CCI Rodents

Von Frey paw withdrawal thresholds (PWTs) are presented as mean (±SEM). The Mann–Whitney nonparametric test, as implemented in the GraphPad Prism™ statistical analysis program (v5.03, GraphPad Software Inc, La Jolla, CA, USA) was used to compare PWT values between the various genotypes for CCI mice herein and for CCI rats prior to administration of EMA300 and at 1 hour postdosing. The statistical significance criterion was $P < 0.05$.

Image Analysis

For quantitative assessment of the extent of immunoreactive cells in the DRGs, sections stained on the same day were photographed in parallel using the same camera settings. At least four sections (>100 μM apart) from each DRG (L4-L6) for each animal (N = 4–6) were analyzed and a mean level of fluorescence determined. Software was used to set the upper and lower threshold values of IF.

Only whole, intact neurons were included in the analysis, and the ratio of immunofluorescent positive pixels versus the total number of pixels for each section determined. Results are expressed as the fold change from nonoperated animals. All analysis was carried out in a double-blinded manner. The statistical significance criterion was $P < 0.05$. The Mann–Whitney nonparametric test, as implemented in the GraphPad Prism statistical analysis program (v5.03) was used to compare the level of IF between groups.

Results

Genetic Deletion of the AT_2R Abolishes EMA300 Analgesia in CCI Mice

Following CCI surgery in female wild-type, AT_2R KO and hemizygous mice, mechanical allodynia developed to a similar extent in the ipsilateral hindpaws irrespective of genotype. Specifically, the mean ($\pm$SEM) von Frey PWT values for the ipsilateral hindpaws decreased
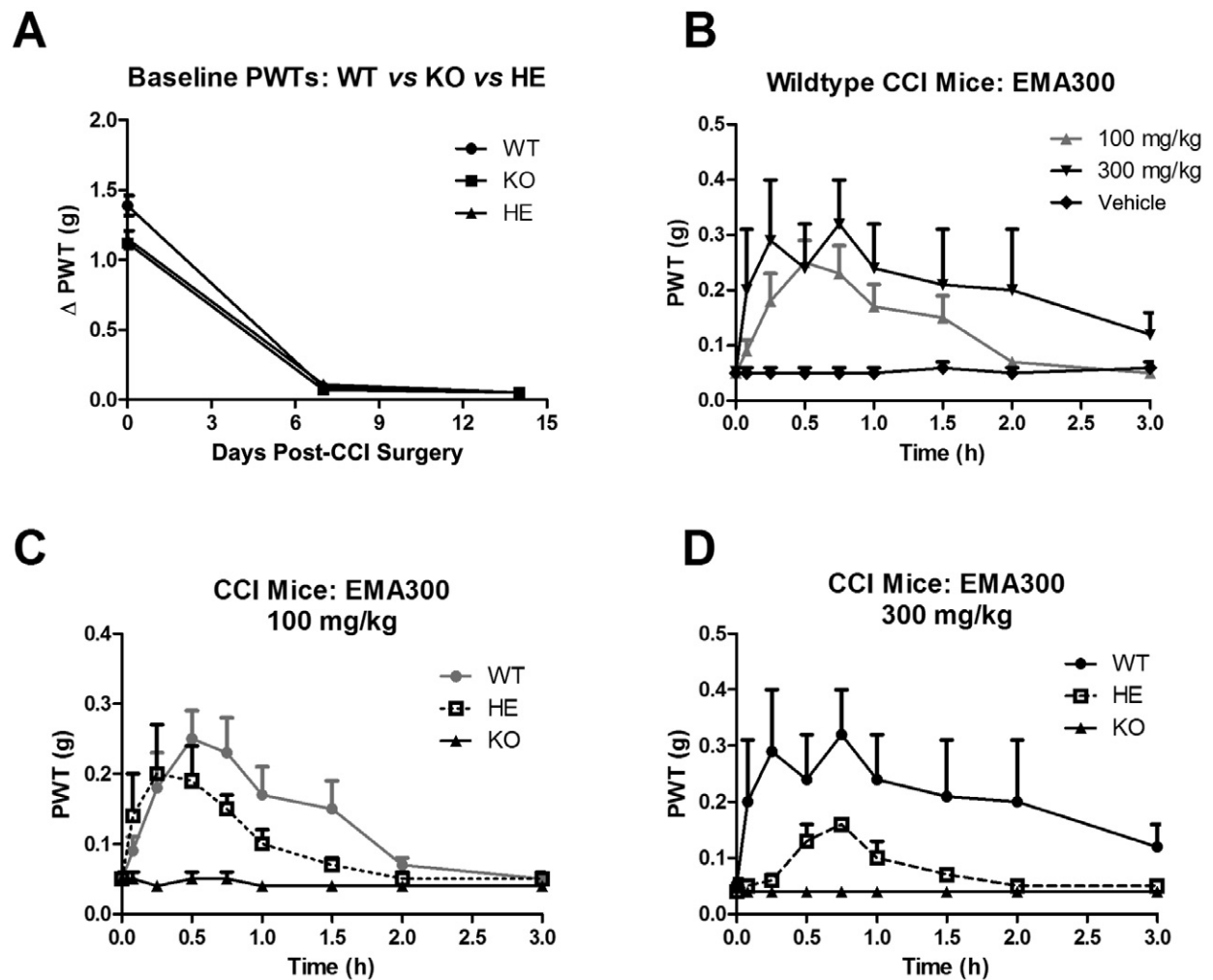
Figure 2 EMA300 analgesia is abolished in AT_2R knockout CCI mice. In CCI mice, the extent of mechanical hypersensitivity that developed in the ipsilateral hindpaws did not differ significantly between wild-type (WT), AT_2R knockout (KO), and hemizygous (HE) mice (A). Single i.p. bolus doses of EMA300 at 100 (N = 8) and 300 mg/kg (N = 3) produced dose-dependent relief of mechanical allodynia in the ipsilateral hindpaws of wild-type CCI mice, whereas vehicle was inactive (N = 5) (B). The analgesic effects of EMA300 at 100 mg/kg (N = 6) and 300 mg/kg (N = 3) were abolished in AT_2R KO CCI mice and intermediate responses were evoked in the corresponding hemizygotes (C, D respectively). CCI = chronic constriction injury.

significantly ($P < 0.05$) from 1.39 (± 0.07) g presurgery to 0.09 (± 0.02) g by 7 days post-CCI surgery in wild-type mice (Figure 2A). The corresponding pre- and day 7 post-CCI surgery PWT values for the ipsilateral hindpaws of AT_2R-KO and hemizygous mice were 1.12 (± 0.04) g to 0.07 (± 0.02) g and 1.15 (± 0.06) g to 0.11 (± 0.02), respectively (Figure 2A). At day 14 post-CCI surgery, the mean ($\pm$SEM) ipsilateral PWT values were 0.05 (± 0.01) g for wild-type mice, 0.05 (± 0.00) g for AT_2R KO mice, and 0.05 (± 0.01) g for the hemizygotes, respectively, demonstrating that there was no significant difference ($P > 0.05$) between the genotypes (Figure 2A).

Administration of single i.p. bolus doses of EMA300 at 100 and 300 mg/kg produced dose-dependent relief of mechanical allodynia in the ipsilateral hindpaws of wild-type CCI-mice. Peak effects were observed at 30–45 minutes postdosing (Figure 2B), whereas vehicle was inactive. At the highest dose tested, the corresponding duration of action was >3 hours. In homozygous AT_2R KO CCI mice, the analgesic effects of EMA300 at 100 and 300 mg/kg were abolished, whereas intermediate responses were produced in the hemizygotes (Figure 2C,D), thereby demonstrating a gene dose–response relationship. In the doses tested in CCI mice, EMA300 did not produce overt behavioral side effects, irrespective of genotype.

Effect of Analgesic Dose of EMA300 on Lumbar DRG Levels of Angiotensin II, pp38 and pp44/pp42 MAPK in CCI Rats
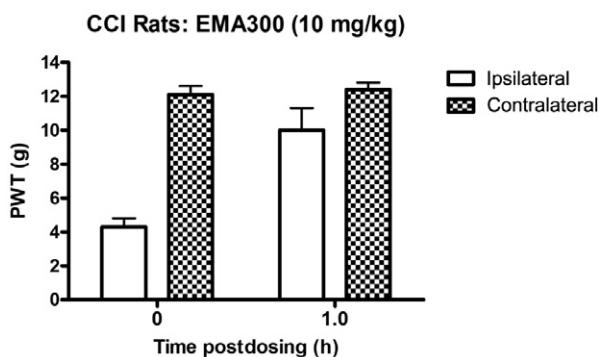
In rats with fully developed mechanical allodynia in the ipsilateral hindpaws at 14-days post-CCI surgery, single bolus intraperitoneal doses of EMA300 at 10 mg/kg alleviated ipsilateral hindpaw hypersensitivity at 1 hour postdosing (Figure 3), in a manner similar to that reported previously by our laboratory in CCI rats [3].

pp38 MAPK and pp44/p42 MAPK

Mean levels of IF for the putative chronic pain markers, pp38 MAPK and pp44/pp42 MAPK in the ipsilateral lumbar (L4-L6) DRGs of CCI rats with fully developed mechanical allodynia in the ipsilateral hindpaw at 14-days post-CCI surgery, were 1.5- to 2.0-fold higher ($P < 0.05$) than for the corresponding sham-operated animals (Figure 4A,B). At the time of peak pain relief (1 hour postdosing) of EMA300 at 10 mg/kg in CCI rats, mean ipsilateral lumbar DRG levels of pp38 MAPK and pp44/pp42 MAPK were reduced ($P < 0.05$) to match the respective levels in sham controls (Figure 4A,B).

Angiotensin II

At 14-days after CCI and sham surgery in rats, mean ($\pm$SEM) levels of angiotensin II IF were significantly ($P < 0.05$) increased in the ipsilateral lumbar DRGs at 376 (± 12)% and 220 (± 31)% relative to the corresponding levels for nonoperated animals (arbitrarily set at 100%)

CCI Rats: EMA300 (10 mg/kg)

Figure 3 EMA300 produces analgesia in rats with peripheral-nerve injury-induced neuropathic pain. In CCI rats with fully developed mechanical allodynia in the ipsilateral hindpaws at 14-days post-CCI surgery, single i.p. bolus doses of the AT_2R antagonist, EMA300 at 10 mg/kg, alleviated mechanical allodynia at the time of peak effect (1 hour postdosing). CCI = chronic constriction injury.

(Figure 4C). Although the mean levels of angiotensin II IF were ~twofold higher in CCI cf. sham-controls, the mean level of angiotensin II IF at 349 (± 7)% was not significantly altered ($P > 0.05$) in the ipsilateral lumbar (L4-L6) DRGs by a single bolus i.p. dose of EMA300 (10 mg/kg) at the time of peak effect (1 hour postdosing) relative to that determined for untreated CCI rats (Figure 4C).
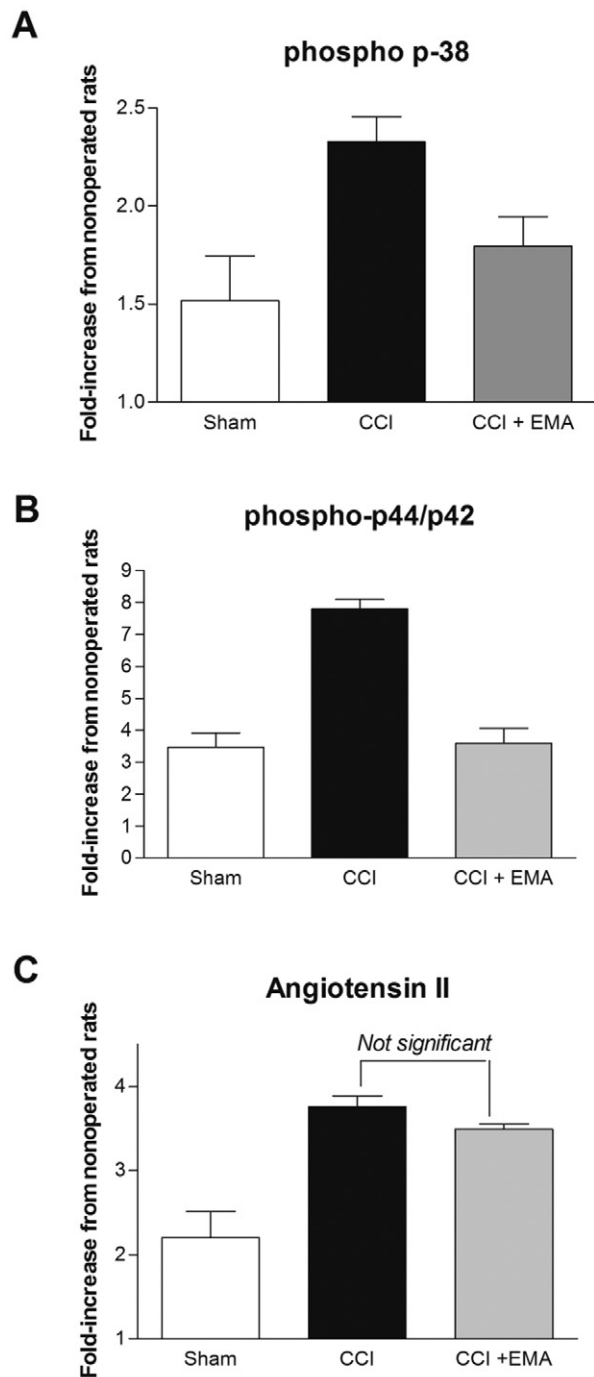
Immunohistology

Specificity of Abcam AT_2R Antibody (ab 19134)

The specificity of the immunofluorescent (IF) Abcam AT_2R antibody (ab 19134) was demonstrated in sections of adrenal gland and lumbar DRGs from adult female mice, as the fluorescence observed in wild-type mouse sections (Figure 5A,B) was abolished in the corresponding sections from AT_2R KO mice (Figure 5E,F). By contrast, the IF Santa Cruz AT_2R antibody (sc-9040) was found to be nonspecific in sections of adrenal gland (Figure 5C,D) and lumbar DRGs (Figure 5G,H).

Colocalization of the AT_2R and Angiotensin II with Sensory Neuron Markers in Rat DRGs

Using specific IF-labeled antibodies in sections of lumbar DRGs from CCI, sham-control and nonoperated rats, we show that the AT_2R (Figure 6A) and angiotensin II (Figure 7A) are strongly colocalized with substance P (Figures 6B, 7B), a marker of small/medium diameter nociceptive neurons (Figures 6C, 7C) in adult rat lumbar DRGs. Additionally, we show that AT_2R IF (Figure 6G) is colocalized with that for NGF (Figure 6H,I) and its high affinity receptor, TrkA, (Figure 6J,N, respectively). AT_2R-IF

A

phospho p-38

B

phospho-p44/p42
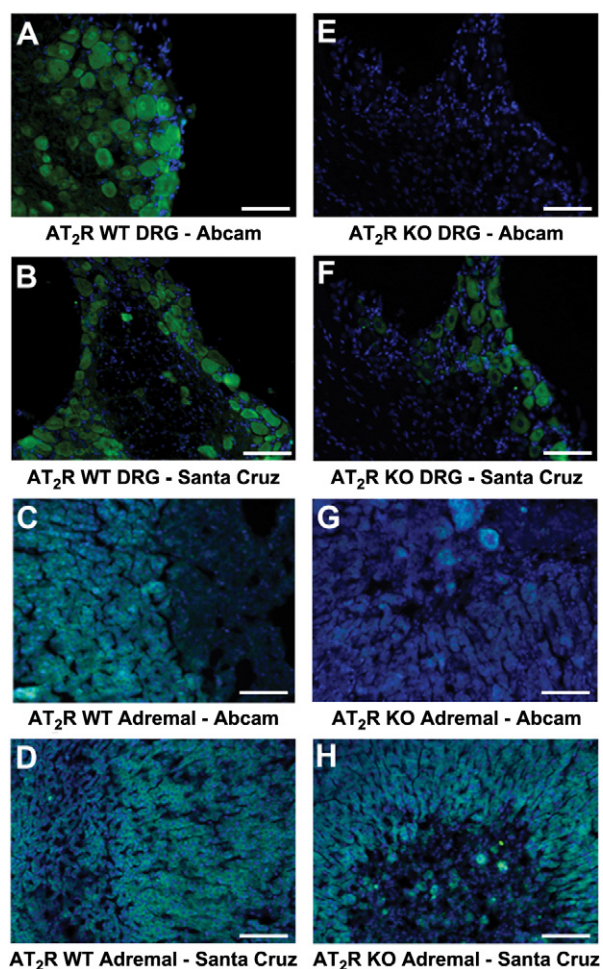

C

Angiotensin II

Figure 4 Analgesic doses of EMA300 inhibited p38 MAPK and p44/p42 MAPK activation in the lumbar DRGs of CCI rats. In CCI rats, there was a significant increase ($P < 0.05$) in the mean ($\pm$SEM) ipsilateral lumbar DRG levels of immunofluorescence for pp38 MAPK (A), pp44/pp42 MAPK (B), and angiotensin II (C) compared with the corresponding levels for sham-controls at 14-days post-CCI surgery. At the time of peak EMA300 analgesia (1 hour postdose) in CCI rats, mean ($\pm$SEM) ipsilateral lumbar DRG levels of pp38 MAPK and pp44/pp42 MAPK were reduced significantly ($P < 0.05$) to match the corresponding levels for sham-controls (N = 5–6 rats per group). CCI = chronic constriction injury; DRG, dorsal root ganglion; MAPK, p38 mitogen-activated protein kinase.

Discussion

Small molecule AT$_2$R antagonists with >1,000-fold specificity over the AT$_1$R were reported recently by our laboratory to produce dose-dependent analgesia in CCI rats [3], a widely utilized rat model of neuropathic pain. Here we used mice with genetic deletion of the AT$_2$R to confirm the AT$_2$R as the target mediating EMA300 analgesia in the CCI mouse model of neuropathic pain. Specifically, our data show a gene dose-related reduction in EMA300 analgesia in CCI mice such that genetic deletion of the AT$_2$R abolished analgesia and intermediate responses were produced in the hemizygotes (Figure 2). Additionally, we show an absence of AT$_2$R IF in the DRGs and adrenal glands of AT$_2$R knockout (KO) mice (Figure 5). Interestingly, the extent to which mechanical hypersensitivity developed in the hindpaws of CCI mice herein did not differ significantly ($P > 0.05$) between wild-type, AT$_2$R KO, and hemizygous animals (Figure 2), suggesting that angiotensin II signaling via the AT$_2$R may be more important in the maintenance rather than the establishment phase of neuropathic pain.

In CCI rats with fully developed mechanical allodynia in the ipsilateral hindpaws herein, single bolus doses of EMA300 at 10 mg/kg fully reversed hindpaw hypersensitivity at the time of peak effect at 1 hour postdosing (Figure 3) in agreement with recent findings from our laboratory [3]. In these CCI rats, there was an ~2-fold increase in mean levels of angiotensin II IF cf. sham-operated rats that was not significantly altered ($P > 0.05$) at the time of peak effect (1 hour postdosing) of an analgesic dose of EMA300 at 10 mg/kg (Figure 4C). By contrast, mean ipsilateral lumbar DRG levels of activated p38 MAPK and p44/p42 MAPK IF that were ~1.5- to 2.0-fold higher in CCI rats cf. the respective levels in sham-controls, were reduced to match the levels in sham-controls at the time of peak

was strongly colocalized with neurofilament 200 (NF200) (Figure 6F), a marker of medium-/large-diameter neurons, whereas angiotensin II was colocalized with only a subset of NF200-positive cells (Figure 7F). The level of AT$_2$R-IF in the ipsilateral lumbar DRGs of neuropathic rats (Figure 6K) appeared to be reduced relative to the corresponding levels observed for sham-control animals (Figure 6G).

Figure 5 Specificity of the AT_2R antibody. Sections of adult female mouse DRG and adrenal gland from wild-type (A,B) and AT_2R knockout mice (E,F) show that the Abcam anti-AT_2R antibody ab 19134 immunofluorescence is specific for the AT_2R, whereas immunofluorescence produced by the Santa Cruz anti-AT_2R antibody sc-9040 is nonspecific in both adrenal gland (C,D) and lumbar DRG (G,H) sections. Bar = 50 μm. CCI = chronic constriction injury; DRG, dorsal root ganglion.

EMA300 analgesia (Figure 4A,B). Together, our data show that the mode of action through which EMA300 produces analgesia in the CCI rat model of neuropathic pain appears to involve attenuation of augmented angiotensin II signaling via the AT_2R in the DRGs to inhibit p38 MAPK and p44/p42 MAPK activation. This mechanism for EMA300 analgesia in neuropathic pain is supported by previous work by others showing that inhibition of p38 MAPK and p44/p42 MAPK activation in lumbar DRGs of CCI rats produces analgesia in these animals [23,24].

Clinical validity for targeting inhibition of MAPK activation as a strategy for discovery of new analgesics for relief of intractable neuropathic pain comes from studies showing high expression levels of pp38 MAPK and pp44/pp42 MAPK in painful neuromas in humans [19] and experimental neuromas in rats [16]. In an exploratory, crossover, placebo-controlled clinical study, administration of the oral p38 MAPK inhibitor, dilmapimod at 7.5 mg/kg twice-daily, produced significant analgesia above placebo in patients with neuropathic pain and was well-tolerated [20,21]. By contrast, although 4 weeks treatment with the oral p38 MAPK inhibitor, losmapimod at either 7.5 mg once- or twice-daily was well-tolerated, insignificant analgesa above placebo was produced in similar patients with neuropathic pain [25]. Whether the conflicting pain relief outcomes between dilmapimod and losmapimod is influenced by between-agent differences in exposure at the target site or specificity for inhibition of specific p38 MAPK isoforms is unclear [25]. Over the past two decades, multiple clinical trials have investigated the safety and efficacy of p38 MAPK inhibitors in a range of inflammatory disease conditions, including rheumatoid arthritis, Crohn's disease, asthma, and psoriasis, with several trials being terminated early for toxicity reasons (see [26] for review). More recently, it has been appreciated that a potentially attractive strategy for achieving the desired efficacy while minimizing adverse events evoked by direct p38 MAPK inhibition is to develop drugs that modulate targets that regulate p38 MAPK function [26]. To this end, our findings herein show that the small molecule AT_2R antagonist, EMA300, blocked peripheral nerve injury-induced augmentation of angiotensin II/AT_2R signaling in the ipsilateral DRGs of nerve-injured rats, resulting in downstream inhibition of p38 MAPK and p44/p42 MAPK activation in the DRGs, with the net result being pain relief. Inhibition of p38 MAPK and p44/p42 MAPK activation in the DRGs would be expected to reduce phosphorylation of multiple receptors and ion channels implicated in DRG neuronal hyperexcitability and neuropathic pain. These include major voltage-gated sodium and calcium channels, such as $Na_v1.8$ [14], $Na_v1.7$ [15,16], $Ca_v2.2$ [17], as well as TRPV1 [18]. Furthermore, prevention of p38 MAPK activation in the ipsilateral lumbar DRGs of CCI rats by EMA300 will attenuate nerve growth factor (NGF)-induced increases in TRPV1 expression levels in the DRGs that would otherwise occur [18,27].

A role for augmented angiotensin II/AT_2R signaling to induce sensory neuron hyperexcitability in neuropathic pain by a mechanism involving phosphorylation of the TRPV1 receptor is supported by recent work showing that angiotensin II increased capsaicin (prototypic TRPV1 agonist)-evoked excitatory responses in cultured adult rat and human DRG sensory neurons, with these effects reduced in a concentration-dependent manner by EMA401, an orally active small molecule AT_2R antagonist [5]. In the same study, angiotensin II induced neurite outgrowth in cultured adult rat and human DRG sensory neurons, with this effect also attenuated by EMA401 in the absence of neurotoxicity [5]. These latter findings [5] extend earlier work by others in cultured cells of neuronal
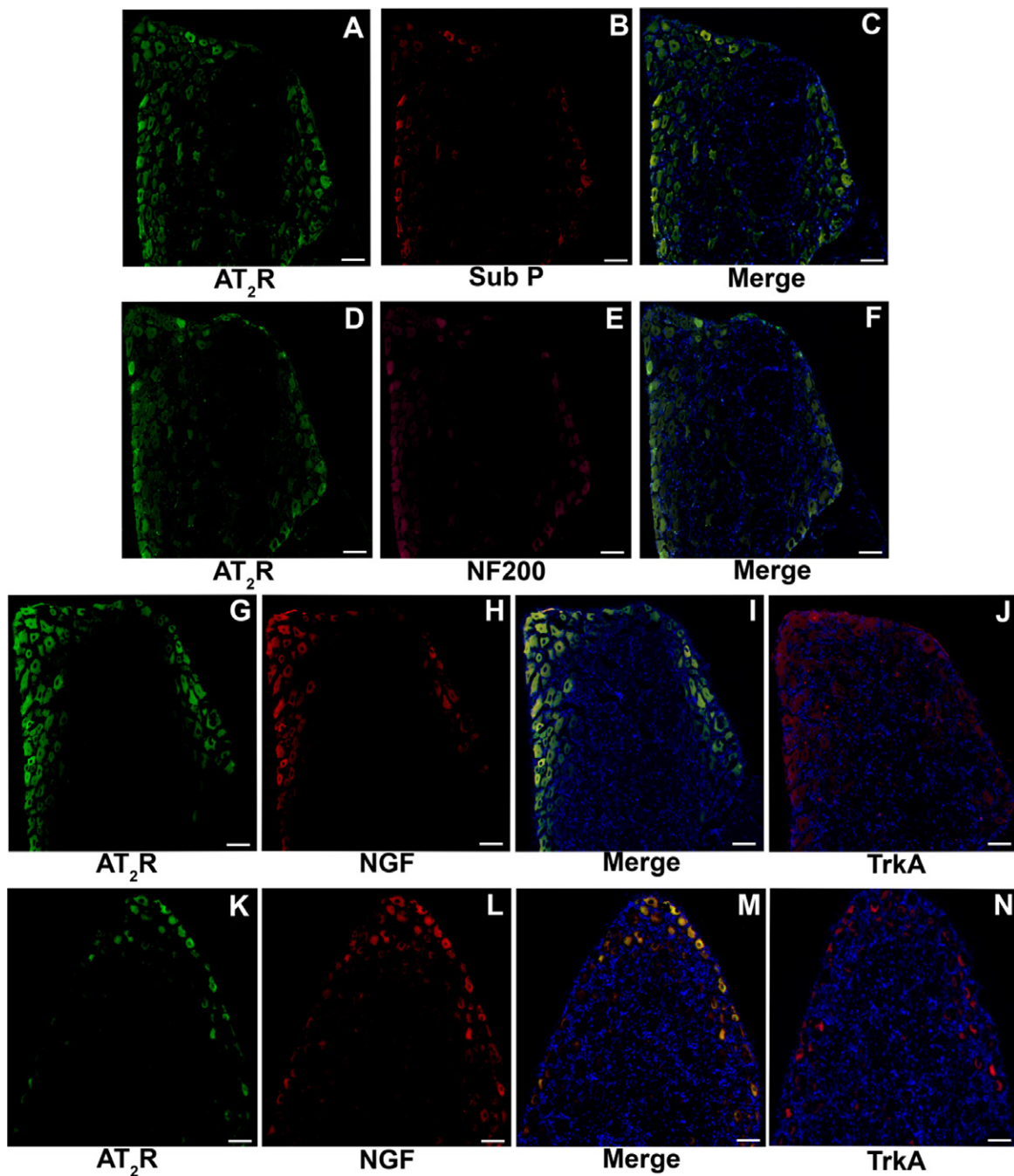
Figure 6 AT$_2$R expression in adult rat DRGs. Specific immunofluorescence (IF)-labeled antibodies show that in adult rat lumbar DRG neurons (A), the AT$_2$R (green; Ab 19134) and (B) substance P (red) are (C) colocalized (yellow), and that (D) the AT$_2$R (green) and (E) neurofilament 200 (NF200) (red) are (F) colocalized (yellow). The AT$_2$R (G,K) was strongly colocalized (yellow) with NGF (H,L) in ipsilateral lumbar DRGs from sham-control (I) and CCI rats (M), respectively. Consecutive ipsilateral lumbar DRG sections of sham-control and CCI rats exposed to specific IF-labeled antibodies for the AT$_2$R alone (G,K, respectively) and the high affinity NGF receptor, Trk A, alone (J,N, respectively) indicate likely colocalization of the AT$_2$R and the high affinity NGF receptor, TrkA. Levels of AT$_2$R IF were consistently lower in the ipsilateral lumbar DRGs of CCI rats (K) cf. sham controls (G). Bar = 50 µm. CCI = chronic constriction injury; DRG, dorsal root ganglion.
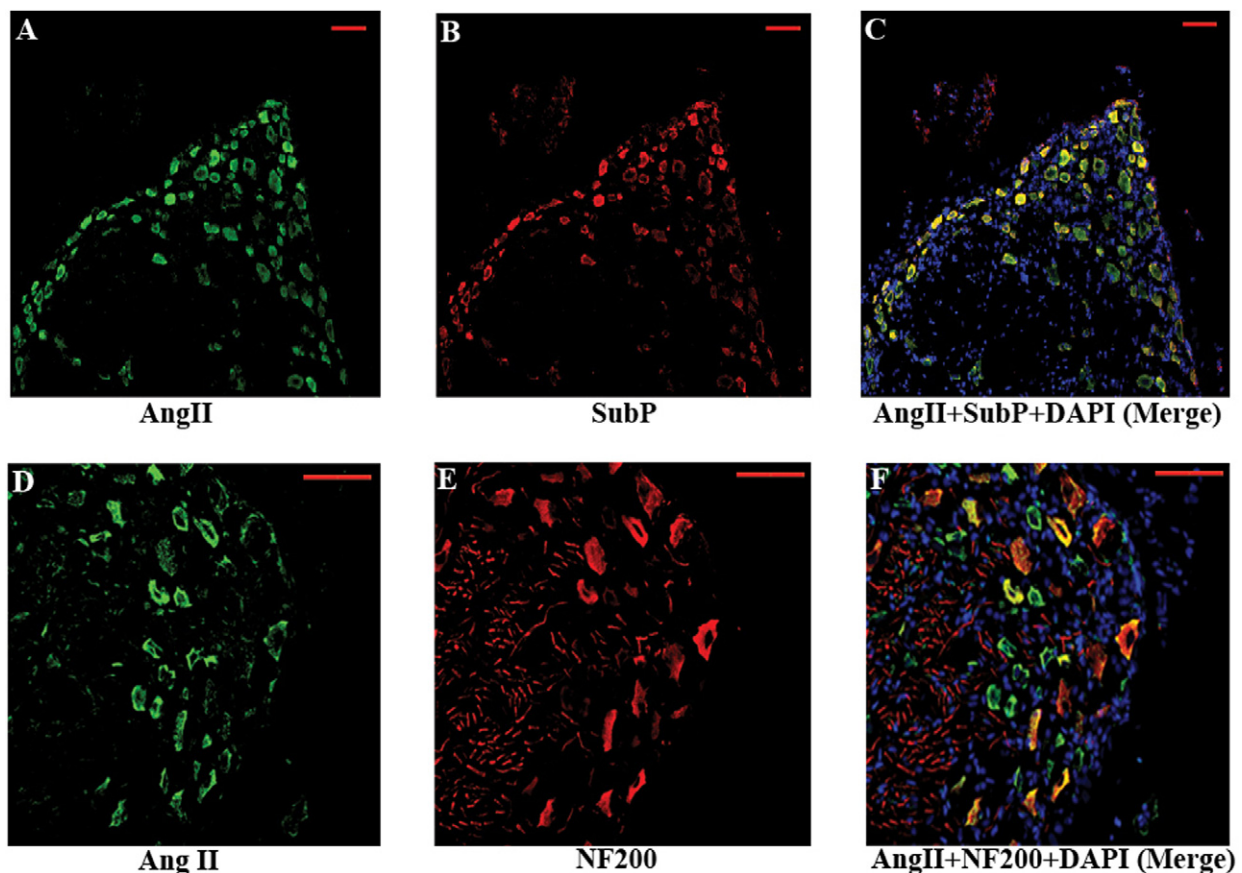
Figure 7 Angiotensin II expression in adult rat DRGs. Specific IF-labeled antibodies show that in adult rat lumbar DRG neurons, (A) angiotensin II (green) and (B) substance P (red) are (C) colocalized (yellow), whereas (D) angiotensin II (green) and (E) neurofilament 200 (NF200) (red) are (F) colocalized (yellow) in a subset of neurons. DAPI (blue) binds to all cellular DNA and is a general marker for the presence of cells. Bar = 50 μm. CCI = chronic constriction injury; DRG, dorsal root ganglion; IF = immunofluorescence.

origin (NG10815 and PC12W cells) [12,13,28], in rat cerebellar granule neurons [29], and in rat brain [6] and DRG neurons [7], showing that angiotensin II signaling via the AT_2R induced neuronal excitability [6] and neurite outgrowth [7,13,28].

Using immunohistochemical methods in sections of adult rat lumbar DRGs herein, we show strong colocalization of the AT_2R (Figure 6) and angiotensin II (Figure 7) with substance P and NGF that are markers of small-/medium-diameter nociceptive neurons. Our findings agree with previous findings of the colocalization of Ang II with the small-/medium-diameter nociceptive neuronal markers, substance P and calcitonin gen-related peptide in adult rat and human DRG neurons [30]. Additionally, our findings are aligned with the presence of AT_2R mRNA in adult rat DRG neurons [30] and the fact that ~60% of small-/medium-diameter neurons in human postmortem and avulsion-injured DRGs are immune-positive for the AT_2R [5]. In the present study, we also found strong

colocalization of the AT_2R with NF200 (Figure 6F), a marker of medium- to large-diameter neurons, together with the presence of angiotensin II in a subset of NF200-positive cells (Figure 7F) in adult rat lumbar DRGs. These findings are consistent with the notion of a role for augmented angiotensin II/AT_2R signaling in abnormal hyperexcitability of large diameter Aβ-fibers that also occurs in neuropathic pain [31].

Interestingly, levels of AT_2R-IF in sections of ipsilateral lumbar DRGs of CCI rats herein (Figure 6K) appeared to be reduced relative to the respective levels in the lumbar DRGs of sham-controls (Figure 6G) in a manner reminiscent of reports of reduced $Na_v1.8$ sodium channel expression levels in injured afferents in the DRGs of nerve-injured rodents, with expression levels preserved in adjacent non-injured afferents [32–34]. Furthermore, our findings are aligned with the reduced levels of AT_2R-IF in peripheral nerve segments proximal to injury of human limbs, whereas AT_2R-IF levels were preserved in human

neuromas [5] that also express high levels of activated p38 MAPK and p44/p42 MAPK [19].

In cultured NG10815 cells, angiotensin II signaling via the AT$_2$R was found to induce phosphorylation of TrkA independently of NGF to produce sustained activation of p44/p42 MAPK and neurite outgrowth [13], and that NGF-induced neurite outgrowth in cultured adult rDRG neurons was unaffected by AT$_2$R antagonism [7]. Colocalization of the AT$_2$R with NGF and its high affinity receptor, TrkA, in adult rat lumbar DRGs herein is of interest as NGF-mediated activation (phosphorylation) of TrkA potentiates nociceptive signaling via multiple mechanisms [35]. Additionally, clinical studies show that NGF antibodies that block NGF signaling produce excellent analgesia in patients with chronic osteoarthritis pain [36]. Whether AT$_2$R antagonists reduce phosphorylation of TrkA in the DRGs independently of NGF blockade, to inhibit p38 and p44/p42 MAPK activation and reduce hyperexcitability of sensory DRG neurons to produce analgesia in neuropathic pain, remains for future investigation.

Conclusion

In summary, genetic deletion of the AT$_2$R abolished EMA300 analgesia in the CCI mouse model of neuropathic pain, affirming the AT$_2$R as the target mediating EMA300 pain relief. In the CCI rat model of neuropathic pain, augmented angiotensin II/AT$_2$R signaling in the DRGs was blocked at the time of peak effect of an analgesic dose of EMA300. This in turn inhibited p38 MAPK and p44/p42 MAPK activation in the ipsilateral lumbar DRGs of nerve-injured rats at the time of peak EMA300 analgesia. The orally active AT$_2$R antagonist, EMA401, is in clinical development as a novel analgesic for the relief of neuropathic pain.

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