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Sustained stimulation of \( \beta_2 \)- and \( \beta_3 \)-adrenergic receptors leads to persistent functional pain and neuroinflammation

Xin Zhang\(^{a,b,1}\), Jane E. Hartung\(^{a,c,1}\), Andrey V. Bortsov\(^{a}\), Seuntgae Kim\(^{a,d}\), Sandra C. O’Buckley\(^{a}\), Julia Kozlowska\(^{a}\), Andrea G. Nackley\(^{a,x}\)

\(^{a}\) Center for Translational Pain Medicine, Department of Anesthesiology, Duke University School of Medicine, Durham, NC, USA
\(^{b}\) Pain Management Center, Shanghai Jiao Tong University Affiliated Sixth People’s Hospital, Shanghai, China
\(^{c}\) Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA
\(^{d}\) Division of Meridian and Structural Medicine, School of Korean Medicine, Pusan National University, Republic of Korea

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ABSTRACT

Functional pain syndromes, such as fibromyalgia and temporomandibular disorder, are associated with enhanced catecholamine tone and decreased levels of catechol-O-methyltransferase (COMT; an enzyme that metabolizes catecholamines). Consistent with clinical syndromes, our lab has shown that sustained 14-day delivery of the COMT inhibitor OR486 in rodents results in pain at multiple body sites and pain-related volitional behaviors. The onset of COMT-dependent functional pain is mediated by peripheral \( \beta_2 \)- and \( \beta_3 \)-adrenergic receptors (\( \beta_2 \)- and \( \beta_3 \)-ARs) through the release of the pro-inflammatory cytokines tumor necrosis factor \( \alpha \) (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Here, we first sought to investigate the role of \( \beta_2 \)- and \( \beta_3 \)-ARs and downstream mediators in the maintenance of persistent functional pain. We then aimed to characterize the resulting persistent inflammation in neural tissues (neuroinflammation), characterized by activated glial cells and phosphorylation of the mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase (ERK). Separate groups of rats were implanted with subcutaneous osmotic mini-pumps to deliver OR486 (15 mg/kg/day) or vehicle for 14 days. The \( \beta_2 \)-AR antagonist ICI118551 and \( \beta_3 \)-AR antagonist SR59230A were co-administrated subcutaneously with OR486 or vehicle either on day 0 or day 7. The TNFα inhibitor Etanercept, the p38 inhibitor SB203580, or the ERK inhibitor U0126 were delivered intrathecally following OR486 cessation on day 14. Behavioral responses, pro-inflammatory cytokine levels, glial cell activation, and MAPK phosphorylation were measured over the course of 35 days. Our results demonstrate that systemic delivery of OR486 leads to mechanical hypersensitivity that persists for at least 3 weeks after OR486 cessation. Corresponding increases in spinal TNFαs, IL-1β, and IL-6 levels, microglia and astrocyte activation, and neuronal p38 and ERK phosphorylation were observed on days 14–35. Persistent functional pain was alleviated by systemic delivery of ICI118551 and SR59230A beginning on day 0, but not day 7, and by spinal delivery of Etanercept or SB203580 beginning on day 14. These results suggest that peripheral \( \beta_2 \)- and \( \beta_3 \)-ARs drive persistent COMT-dependent functional pain via increased activation of immune cells and production of pro-inflammatory cytokines, which promote neuroinflammation and nociceptor activation. Thus, therapies that resolve neuroinflammation may prove useful in the management of functional pain syndromes.

1. Introduction

Functional pain syndromes (FPS), including fibromyalgia (FM), temporomandibular disorder (TMD), tension-type headache (TTH), and irritable bowel syndrome (IBS), represent a significant healthcare problem that affect nearly one in every three Americans (Clauw, 2014; Dzau and Pizzo, 2014; Rey and Talley, 2009; Slade et al., 2013; Tsang et al., 2008; Wesselmann et al., 2014). These conditions are characterized by persistent pain in the absence of tissue damage and often co-occur, thereby affecting multiple body sites. Accumulating evidence suggests that the origins of FPS are linked to abnormalities in catecholaminergic tone. Patients with FPS have increased levels of the catecholamines epinephrine and norepinephrine (Bote et al., 2014; Evaskus and Laskin, 1972; Perry et al., 1989; Torpy et al., 2000).

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alongside decreased levels of catechol-O-methyltransferase (COMT) (Marbach and Levitt, 1976; Smith et al., 2014), a ubiquitously expressed enzyme that metabolizes catecholamines (Mannisto and Kaakkola, 1999). Consistent with clinical findings, rodents receiving sustained delivery of the COMT inhibitor OR486 exhibit heightened mechanical sensitivity at multiple body sites, including the hindpaw and the abdomen (Ciszek et al., 2016; Kline et al., 2015). The development of this COMT-dependent functional pain is mediated by stimulation of peripheral β2- and β3-adrenergic receptors (β2- and β3ARs) (Ciszek et al., 2016).

β2- and β3ARs are G protein-coupled receptors (GPCRs) implicated in the transmission of pain. β2- and β3ARs can directly produce pain by increasing the excitability of primary afferent nociceptors on which they reside (Aley et al., 2001; Favaro-Moreira et al., 2012; Kanno et al., 2010; Khasar et al., 1999a,b; 2003; Zhang et al., 2014). β2- and β3ARs can also influence pain through activation of immunoregulatory cells. Increased catecholamine levels following stress or pharmacologic manipulation lead to β2AR-mediated activation of T-cells (Laukova et al., 2012; Slota et al., 2015), mast cells (Chi et al., 2004), and macrophages (Chiarella et al., 2014; Kim et al., 2014) and β3AR-mediated activation of adipocytes (Fu et al., 2007; Kralsch et al., 2006; Mohamed-Ali et al., 2001), so as to increase the production of pro-inflammatory cytokines that sensitize nociceptors (Binshtok et al., 2008; Czeschik et al., 2008; Obreja et al., 2002, 2005). Previously, our group demonstrated that acute delivery of OR486 leads to β2- and β3AR-mediated increases in plasma levels of the pro-inflammatory cytokines tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6) within 3 h and that these cytokines were required for the development of functional pain (Hartung et al., 2014).

While acute increases in pro-inflammatory cytokines elicit acute pain and promote tissue repair, sustained elevations elicit persistent pain and maladaptive tissue pathology (Bennett and Schulz, 1993; Dinarello et al., 1990; Gharaei-Kermani and Phan, 2001). Sustained peripheral inflammation leads to increased release of excitatory neurotransmitters from the central terminals of primary afferents in the spinal cord and increased phosphorylation of mitogen-activated protein kinases (MAPKs), producing a state of neuronal hyperactivity in central pain-coding pathways (Woelf, 1983; Woelf and Salter, 2000). This state of ‘central sensitization’ is associated with inflammation in neural tissues (neuroinflammation), characterized by increased activation of glial cells and production of pro-inflammatory cytokines in neural tissues (Ji et al., 2016).

The present study sought to determine the relevance of these neuro-immune interactions to the maintenance of functional pain. We hypothesized that β2- and β3ARs drive persistent COMT-dependent functional pain via increased activation of immune cells and production of pro-inflammatory cytokines, which promote neuroinflammation and nociceptor activation. To test this hypothesis, we measured circulating and spinal levels of pro-inflammatory cytokines; activation of spinal microglia, astrocytes, and neurons; phosphorylation of spinal p38 and ERK MAPKs; and functional pain over the course of 35 days in rats receiving sustained subcutaneous delivery of β2- and β3AR antagonists on day 0 prior to or on day 7 following subcutaneous delivery of OR486 for 14 days. In addition, separate groups received sustained intrathecal delivery of TNFα, p38, or ERK inhibitors on day 14 following OR486 in order to determine the role of these effectors downstream of β2- and β3ARs in the maintenance of functional pain.

2. Materials and methods

2.1. Subjects

Adult male and female Sprague-Dawley rats (N = 210) were purchased (Charles River Laboratories, USA) and bred in-house. Rats weighed between 200 and 400 g for all experimental studies. Rats had ad libitum access to standard laboratory chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University and the University of North Carolina at Chapel Hill, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. General experimental design

First, after establishing the time course for acute COMT-dependent pain, we determined the effects of sustained β2- and β3AR activation on pain and spinal cord glial and neuronal activity in rats receiving the COMT inhibitor OR486 or vehicle, together with the β2AR-specific antagonist ICI118551 + the β3AR-specific antagonist SR59230A or vehicle over the course of 14 days. Pain behavior was measured prior to (day 0), during (days 1, 3, 7, and 14), and 1–3 weeks following (days 21, 28, and 35) drug delivery. Spinal cord glial and MAPK activity were measured in separate groups of rats on day 14 during drug delivery or on day 21 (1 week) following drug delivery.

Next, we evaluated the contribution of β2- and β3ARs to the maintenance of pain and spinal cord glial and neuronal activity in rats receiving ICI118551 + SR59230A or vehicle on day 7 following sustained delivery of OR486 or vehicle. Pain behavior was measured prior to (day 0), during (days 1, 3, 7, and 14), and 1–3 weeks following (days 21, 28, and 35) OR486 or vehicle delivery. Spinal cord glial and MAPK activity were measured in separate groups of rats on day 21 (1 week) following OR486 or vehicle delivery.

Finally, we evaluated the contribution of the pro-inflammatory cytokine TNFα and the MAPKs p38 and ERK in the maintenance of pain in rats by administering the TNFα inhibitor Etanercept, the p38 inhibitor SB203580, the ERK inhibitor U0126, or vehicle on day 14 following sustained delivery of OR486 or vehicle. Pain behavior was measured prior to (day 0), during (days 1, 3, 7, and 14), and 1–3 weeks following (days 21, 28, and 35) OR486 or vehicle delivery.

2.3. Drug preparation

OR486 (Tocris Bioscience, Bristol, UK) was dissolved in a vehicle consisting of a 5:2:3 ratio of dimethylsulfoxide (DMSO, DMSO Store, Fort Lauderdale, FL, USA), ethanol (Decon Labs Inc, King of Prussia, PA, USA) and 0.9% saline (Hospira, San Jose, CA, USA). ICI118551 and SR59230A (Tocris Bioscience) were dissolved in a vehicle consisting of a 1:4 ratio of DMSO and 0.9% saline. Etanercept (Amgen Inc., Thousand Oaks, CA, USA), SB203580 (EMD Millipore, Burlington, MA, USA), and U0126 (EMD Millipore) were dissolved in a vehicle consisting of a 1:1 ratio of DMSO and 0.9% saline. For acute delivery, OR486 (30 mg/kg) was injected intraperitoneally (ip) as previously described (Hartung et al., 2014). For sustained delivery, drug or vehicle solutions were injected into Alzet osmotic mini-pumps (model 2002; Durect Corporation, Cupertino, CA, USA), which have a 0.5 μl/h infusion rate and a 200 μl reservoir volume. Mini-pumps were placed in 15 mL conical tubes containing sterile 0.9% saline and primed overnight in a dry heat bath (Lab Armor, Cornelius, OR, USA) at 37 °C. Subcutaneous delivery of OR486 (15 mg/kg/day) and ICI118551 (1.5 mg/kg/day) + SR59230A (1.67 mg/kg/day) was at a constant rate over 14 days. Intrathecal delivery of SB203580 (12 μg/day), U0126 (12 μg/ day) and Etanercept (10 μg/day) was at a constant rate over 14 days.

For the maintenance studies, the Lynch method (Lynch et al., 1980) was used to delay delivery of ICI118551 + SR59230A until day 7. Briefly, coiled PE50 polyethylene tubing (Scientific Commodities Inc., Lake Havasu City, AZ, USA) was placed onto the stainless steel flow moderator, which delayed drug delivery according to the length and diameter of the tubing. The pumps filled with Etanercept, SB203580, and U0126 were implanted on day 14, at which time the OR486 and vehicle pumps were removed.
2.4. Surgical procedures

Rats were anesthetized by isoflurane (Baxter, San Juan, Puerto Rico) inhalation (5% induction, 1.5–2% maintenance). Incision sites were shaved and disinfected with 70% ethanol and betadine (CVS Health, Woonsocket, RI, USA). Eyes were lubricated (Lubrifresh PM Ophthalmic Ointment; Major Pharmaceuticals, Livonia, MI, USA). Sterile technique was employed throughout the duration of all procedures according to IACUC requirements. For systemic delivery (Cizzek et al., 2016) of OR486, βAR antagonists, or vehicle, a small incision was made over the left shoulder blade of the rat. Hemostats were used to create a small subcutaneous pocket, in which each pump was placed. Several 9 mm stainless steel wound clips (Stoelting Co, Wood Dale, IL, USA) were used to close the incisions.

For intrathecal delivery (Cizzek et al., 2016) of TNFα, p38, and ERK inhibitors or vehicle, a small incision was made on the nape of the neck, and scissors and hemostats were used to lift muscle and expose the atlanto-occipital membrane. The membrane was carefully incised using the tip of a pair of scissors, a 12.3-cm, polyurethane Alzet Short Rat IT Catheter (Durect Corporation) inserted into the intrathecal space, and placed dorsal to the spinal cord. The catheter was sutured to the surrounding tissue using 4.0 silk suture (Oasis Surgical, Scottsdale, AZ, USA) and attached to the osmotic pump, which was subcutaneously implanted over the right shoulder blade. The initial OR486 or vehicle pumps were removed before incision closure. Delivery of the TNFα, p38, and ERK inhibitors or vehicle lasted for 14 days.

2.5. Assessment of pain behaviors

Paw withdrawal threshold was measured using the von Frey up-down method, as described by Chaplan et al. (1994). Nine calibrated von Frey monofilaments (bending forces of 0.69, 1.2, 1.5, 2.1, 3.6, 5.7, 8.7, 11.7, and 15.0 g; Stoelting) with equal logarithmic spacing between filaments were applied to the plantar surface of the hind paw. A series of 6 applications of monofilaments with varying gram forces was applied to the plantar surface of the hindpaw. Testing began with the middle filament in the series (3.6 g). If the response included the withdrawal of the hindpaw, an incrementally lower filament was applied. In the absence of a paw withdrawal, an incrementally higher filament was applied. These data were entered into the Paw Flick module within the National Instruments LabVIEW 2.0 (Austin, TX, USA) software. Mechanical alldynia was defined as a heightened response to a normally innocuous stimulus and was determined as a significant decrease in paw withdrawal threshold from baseline.

After determining paw withdrawal threshold, paw withdrawal frequency to a noxious von Frey monofilament was assessed. The highest gram force filament (15.0 g) was applied to the hind paw 10 times. Stimulus was applied for 1 s followed by a 1-second interval without a stimulus. The number of paw withdrawals was recorded for each hindpaw. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals to a noxious mechanical stimulus from baseline. In all behavioral experiments, the experimenter was blinded to treatment.

2.6. Assessment of cytokines

Plasma and cerebral spinal fluid (CSF) were collected from separate groups of rats on days 0, 14, 21, and 35 following OR486 delivery. TNFα, IL-1β, and IL-6 were measured using the Rat Cytokine Multiplex Assay from BioRad (Hercules, CA, USA). All plasma samples were diluted at 2× and CSF samples were run neat.

2.7. Assessment of spinal cord glial and neuronal activity

Five minutes prior to tissue collection, mechanical pinch was briefly applied to the hindpaws in order to evoke pERK. Then, rats were deeply anesthetized with Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI, USA) and when no longer responsive to paw- or tail-pinch, they were perfused with 4% paraformaldehyde (PFA; ThermoFisher, Carlsbad, CA, USA) in 0.1 M PBS (Life Technologies, Carlsbad, CA, USA). Spinal cords were collected, placed in 4% PFA post-fixative overnight, and cryoprotected in 30% sucrose (BDH, Kandivali, Mumbai, India) in 0.01 M PBS at 4 °C. Tissues were then embedded in Optimal Cutting Temperature (OCT) cryomatrix and sectioned by cryostat. Spinal cords were sliced to 30-μm-thick free-floating sections, placed in chilled 0.01 M PBS with 0.1% sodium azide, and stored at 4 °C until use.

A combination of antibodies was used to determine glial activation and MAPK phosphorylation in spinal cord. For spinal cord staining, anti-p-p38 (1:300, Cell Signaling, Danvers, MA, USA) or anti-pERK (1:600, Cell Signaling) was used in combination with either anti-CD11b (1:500, AbD Serotec, Hercules, CA, USA), anti-GFAP (1:500, Cell Signaling), or anti-NeuN (1:500, Millipore). Secondary antibodies were either anti-rabbit or anti-mouse Alexa Fluor 488 or Alexa Fluor 594 (Jackson Immuno, West Grove, PA, USA). Stained tissues were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were collected on a Zeiss 780 confocal microscope. Quantification of immunofluorescence and immunopositive cells for spinal cord was conducted using ImageJ/Fiji software (National Institutes of Health, Bethesda, MD, USA).

For p-p38, CD11b, and GFAP analysis, the spinal dorsal horn area was outlined and fluorescence intensity was quantified using Image J software. Data were normalized to the Veh/Veh group and shown as the percentage compared to Veh/Veh. For pERK analysis, the number of pERK+ cells in dorsal horn were counted manually. Control experiments, including incubation of slices in primary or secondary antibody alone, were conducted for each round of staining and showed only low intensity non-specific binding patterns.

2.8. Assessment of MAPK inhibition

Flash frozen spinal cord tissue was homogenized in a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) with tissue protein extraction reagent (TPER, ThermoFisher). To accommodate for variation in tissue homogenates, protein concentrations were normalized following bicinchoninic acid (BCA; Pierce, Grans Island, NY, USA) measurement of protein content to evenly load protein into wells of 10% SDS-PAGE gels (BioRad). Gels were run using standard SDS-PAGE methods and transferred onto PVDF membrane using the iBlot dry blotting system (Life Technologies). Membranes were then blocked, probed with primary antibodies for GAPDH (1:1000; Cell Signaling), p-ERK1/2 (1:1000; Cell Signaling) or p-p38 (1:100; Cell Signaling), and subsequently probed with a corresponding secondary antibody. Specific bands were visualized with enhanced chemiluminescence (Thermo Scientific, Waltham, MA, USA) and quantified with Image J. The relative levels of p-p38 and pERK were normalized to GAPDH.

2.9. Statistical analysis

Group differences in mechanical allodynia and mechanical hyperalgesia over time were analyzed by two-way ANOVA for repeated measures or by one-way ANOVA for the time-collapsed panel insets. Group differences in cellular activity at a single time point were analyzed by one-way ANOVA. Post-hoc comparisons were performed using the Bonferroni test, which corrected for multiple comparisons. Statistical significance was defined as \( P < 0.05 \). All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

Time-dependent changes in plasma vs. CSF concentrations of the cytokines were analyzed using a general linear model as implemented in SAS PROC GLM (SAS 9.4, SAS Institute Inc, Cary, NC). Before the analysis, plasma concentrations for each cytokine were standardized by
subtracting the mean and dividing by the standard deviation. CSF concentrations were standardized in the same way. In the linear model, cytokine concentration was used as a continuous dependent variable. Biomarker source (plasma vs. CSF, coded as a binary variable), batch effect, and time (number of days since the beginning of experiment as a continuous variable), together with source-by-time interaction term, were included as independent variables. The source-by-time interaction term in the model can be interpreted as the difference in time slopes (trends) between standardized plasma and CSF concentrations. A Bonferroni correction was applied to account for testing three cytokines, and the P-value for the interaction term adjusted to a significance threshold of 0.017.

3. Results

3.1. Sustained COMT inhibition results in persistent mechanical allodynia and hyperalgesia mediated by β2- and β3ARs

We first determined the duration of a single ip dose of the COMT inhibitor OR486 (30 mg/kg) on mechanical pain. We found that a single dose of OR486 produced mechanical allodynia (F1, so = 96.27, P < 0.0001) and mechanical hyperalgesia (F1, so = 94.41, P < 0.0001), which peaked at 3 h and returned to baseline at 12 h. (Supplementary Fig. 1). These data suggest that a single dose of COMT inhibitor is not sufficient to drive chronic pain states. As a result, we chose to administer OR486 for continuously over 14 days to assess the effects of sustained reductions in COMT activity. In addition, as the magnitude and duration of mechanical allodynia and hyperalgesia were similar in males and females (P > 0.05), we used male rats for subsequent behavioral experiments.

Our previous work demonstrated that sustained 14-day delivery of the COMT inhibitor OR486 in rats results in mechanical hypersensitivity at multiple body sites, mediated by peripheral β2- and β3ARs (Ciszewski et al., 2016; Kline et al., 2015). Here, we show that this β2- and β3AR-mediated pain persists for at least 3 weeks following OR486 cessation. As shown in Fig. 1, sustained delivery of OR486 resulted in mechanical allodynia (F1,128 = 199.7, P < 0.0001) and mechanical hyperalgesia (F1,128 = 415.7, P < 0.0001) beginning on day 1 and lasting until day 35 (21 days following the cessation of OR486). Co-administration of the β2AR antagonist ICI118551 and the β3AR antagonist SR59230A together with OR486 on day 0, blocked the development of COMT-dependent mechanical allodynia (F3,208 = 64.19, P < 0.0001) and mechanical hyperalgesia (F3,208 = 194.2, P < 0.0001) throughout the 35-day experimental paradigm. The moderate dip in paw withdrawal threshold on days 14 and 28 may be attributed to a lower COMT activity. Co-administration of ICI118551 + SR59230A together with vehicle on day 0 had no effect on pain behavior. Therefore, β2- and β3ARs are required for the onset of mechanical allodynia and hyperalgesia.

3.2. Sustained COMT inhibition results in persistent neuroinflammation.

Our previous work demonstrates that peripheral β2- and β3ARs initiate COMT-dependent functional pain through increased circulating levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 (Hartung et al., 2014). Here, we investigated changes in levels of these pro-inflammatory cytokines in CSF as well as circulating blood collected on day 0, on day 14 following OR486 delivery, and on days 21 and 35 (1 and 3 weeks following OR486 cessation). As shown in Fig. 2, plasma levels of TNFα decreased while CSF levels increased over the course of 35 days, resulting in a significant source-by-time interaction (P = 0.0078). Similarly, opposite trends in levels of IL-1β and IL-6 were observed in plasma versus CSF, resulting in significant source by time interactions (P = 0.021 and P = 0.0098, respectively). Source-by-time interactions for TNFα and IL-6 remained significant after Bonferroni adjustment for three statistical tests. Fig. 2 insets further show the percent change in CSF vs plasma pro-inflammatory cytokine levels on day 35 compared to day 0. By day 35, plasma levels of TNFα, IL-1β, and IL-6 have decreased 30–60%, while CSF levels have increased 50–180%.

These and previous findings, together, suggest that COMT inhibition leads to β2- and β3AR-mediated inflammation in peripheral tissues within hours, that resolves and transitions to neuroinflammation over the course of weeks.

3.3. Sustained COMT inhibition results in β2- and β3AR-mediated increases in the activation of spinal cord microglia and astrocytes

Neuroinflammation is characterized by increased activity of microglia and astrocytes, which are glial cells that contribute to chronic pain following nerve injury or inflammation (Gosselin et al., 2010). Here, we sought to investigate alterations in glial cell activity in our model of functional pain. We first assessed if a single dose of OR486 would lead to glial activation in the dorsal horn. No changes in spinal microglia or astrocyte activation were observed at 3 or 12 h (P > 0.05, Supplementary Fig. 2). However, sustained administration of OR486 for 14 days resulted in increased immunofluorescence of glial markers in the dorsal horn. Compared to vehicle, sustained delivery of OR486 induced microglial activation in the spinal dorsal horn on days 14 (F3,55 = 26.28, P < 0.0001) and 21 (F3,79 = 12.44, P < 0.0001) (Fig. 3A). COMT-dependent increases in microglial activation were blocked by co-administration of ICI118551 + SR59230A on day 0. Similarly, compared to vehicle, sustained delivery of OR486 induced astrocyte activation in the spinal dorsal horn on days 14 (F3,57 = 23.41, P < 0.0001) and 21 (F3,77 = 23.51, P < 0.0001) (Fig. 3B).
(F_{3,74} = 21.27, P < 0.0001) (Fig. 3B). COMT-dependent increases in astrocyte activation were blocked by co-administration of ICI118551 + SR59230A on day 0. Furthermore, we did not observe sex-dependent differences in glial immunoreactivity (Supplementary Fig. 3). Finally, co-administration of ICI118551 + SR59230A in vehicle-treated animals had no effect on the activity of microglia or astrocytes.

3.4. Sustained COMT inhibition results in β2- and β3-AR-mediated increases in the phosphorylation of spinal cord p38 and ERK MAPK

As the p38 and ERK MAPKs are important mediators of pain and inflammation (Ji and Suter, 2007), we sought to investigate their alteration in our model of functional pain. Compared to vehicle, sustained delivery of OR486 induced p38 phosphorylation in the spinal cord on days 14 (F_{1,58} = 9.617, P < 0.0001) and 21 (F_{3,77} = 43.93, P < 0.0001) (Fig. 4A). COMT-dependent increases in p38 phosphorylation were blocked by co-administration of ICI118551 + SR59230A on day 0. Similarly, compared to vehicle, sustained delivery of OR486 resulted in pressure-evoked increases in ERK phosphorylation in the spinal dorsal horn on days 14 (F_{2,28} = 945.34, P < 0.0001) and 21 (F_{2,28} = 1547, P < 0.0001) (Fig. 4B). COMT-dependent pressure-evoked increases in ERK phosphorylation were blocked by co-administration of ICI118551 + SR59230A on day 0. Of note, in the absence of mechanical pinch, spinal ERK phosphorylation was not observed in rats receiving vehicle or OR486 (data not shown). Co-administration of ICI118551 + SR59230A in vehicle-treated animals had no effect on the phosphorylation of p38 or ERK.

To determine the cell types that express p-p38 and pERK in our model of functional pain, we performed subsequent double-labeling immunohistochemical experiments using cell-specific markers for microglia (CD11b), astrocytes (GFAP), and neurons (NeuN). OR486-induced increases in p-p38 and pERK were predominantly found in neurons, 4% in microglia, and 3% in astrocytes (Fig. 5A). Similarly, 87% of pERK was expressed in neurons, 9% in microglia, and 17% in astrocytes (Fig. 5B). Note that percentages do not add up to exactly 100%, as double-staining was performed in alternate spinal cord sections.

3.5. Inhibition of TNFα or p38 reverses COMT-dependent functional pain

The above findings demonstrate that sustained stimulation of β2- and β3ARs results in functional pain and neuroinflammation, characterized by increased pro-inflammatory cytokine production, glial activation, and MAPK phosphorylation in spinal tissues. Next, we targeted β2- and β3ARs and their downstream effectors (TNFα, p38, and ERK) with selective inhibitors to determine the role of these molecules in the maintenance of COMT-dependent functional pain.

To determine the role of β2- and β3ARs in the maintenance of pain, ICI118551 + SR59230A or vehicle were delivered systemically for 14 days, beginning on day 7 following delivery of OR486 or vehicle. Delivery of these β2- and β3AR antagonists on day 7 did not alleviate OR486-induced mechanical allodynia (Fig. 6A) or mechanical hyperalgesia (Fig. 6B). In addition, β2- and β3AR antagonist delivery beginning on day 7 did not reduce OR486-induced glial activation or MAPK phosphorylation (Supplementary Fig. 4).

To determine the role of spinal TNFα in the maintenance of pain, the TNFα inhibitor Etanercept was delivered intrathecally for 14 days, beginning on day 14 (a time point when mechanical sensitivity and neuroinflammation are more established) following delivery of OR486 or vehicle. Results show that Etanercept completely reversed OR486-induced mechanical allodynia (F_{3,151} = 17.62, P < 0.0001; Fig. 6C) and hyperalgesia (F_{3,147} = 30.44, P < 0.0001; Fig. 6D). Similarly, to determine the role of spinal p38 and ERK in the maintenance of pain, the p38 inhibitor SB203580 or the ERK inhibitor U0126 was delivered intrathecally for 14 days, beginning on day 14 following delivery of OR486 or vehicle. Results show that SB203580 reversed OR486-induced mechanical allodynia (F_{3,63} = 16.63, P < 0.0001; Fig. 6E) and hyperalgesia (F_{3,63} = 16.89, P < 0.0001; Fig. 6F). In contrast, U0126 had no effect on OR486-induced pain (Supplementary Fig. 5). The OR486/U0126 group exhibited an increase in paw withdrawal threshold prior to delivery of U0126, making interpretation of the effects of U0126 on mechanical allodynia difficult. Nonetheless, we did find that U0126 had no effect on mechanical hyperalgesia (which was robust and consistent throughout the observation period; Supplementary Fig. 5), suggesting that transient pERK expression is not sufficient to maintain functional pain. Thus, spinal TNFα and p38, which are key mediators of neuroinflammation, are required for the maintenance of functional pain.

To confirm if the doses for SB203580 and U0126 effectively inhibited the phosphorylation of p38 and ERK, respectively, we performed a Western blot to measure p-p38 and pERK. Sustained delivery of OR486 resulted in significant increases in p-p38 expression in the spinal cord on day 35, which was blocked by SB203580. Though OR486 treatment did not increase the pERK expression in spinal cord (in the absence of mechanical stimuli), the ERK inhibitor U0126 decreased pERK expression (Supplementary Fig. 6).
4. Discussion

The present study sought to investigate the role of \( \beta_2 \) - and \( \beta_3 \)ARs and downstream mediators in the maintenance of persistent functional pain linked to abnormalities in catecholamine signaling. Our results are the first to show that sustained stimulation of \( \beta_2 \) - and \( \beta_3 \)ARs produces functional pain and neuroinflammation that persist for weeks after removal of the causal stimulus (systemic COMT inhibitor). Further, we provide evidence that peripherally-initiated functional pain is centrally-maintained by TNF\( \alpha \) and p38 MAPK.

4.1. Sustained stimulation of \( \beta_2 \) - and \( \beta_3 \)ARs leads to persistent mechanical allodynia and hyperalgesia

Pain amplification is a hallmark feature of FPS, with patients experiencing hypersensitivity to mechanical stimuli (Maixner et al., 2016). Mechanical hypersensitivity may occur prior to (predicting onset) or following (signifying chronification) the development of FPS, and often occurs at remote body sites. Consistent with clinical studies, our lab has shown that administration of the COMT inhibitor OR486 in rodents produces increased mechanical hypersensitivity at multiple body sites and alters pain-related volitional behaviors (eg, avoidance of painful heat and bright light) (Ciszek et al., 2016; Hartung et al., 2014; Kline et al., 2015; Nackley et al., 2007). In subsequent pharmacologic studies, we found that the development of acute (measured over 3 h) and persistent (measured over 14 days) OR486-induced mechanical hypersensitivity is mediated by peripheral, but not spinal or supraspinal, \( \beta_2 \) - and \( \beta_3 \)ARs (Ciszek et al., 2016; Hartung et al., 2014; Kline et al., 2015; Nackley et al., 2007; Zhang et al., 2018). This finding is in line with that of Kambur and colleagues, who demonstrated that the peripherally-restricted COMT inhibitor nitecapone elicits mechanical allodynia when administered systemically, but not intrathecally (Kambur et al., 2010). Here, we further demonstrated that systemic delivery of OR486 by osmotic mini-pump for 14 days leads to \( \beta_2 \) - and \( \beta_3 \)AR-mediated mechanical allodynia and mechanical hyperalgesia that persists for at least 3 weeks after OR486 cessation. These findings suggest that sustained increases in catecholamine signaling at peripheral \( \beta_2 \) - and \( \beta_3 \)ARs, strengthen pain-coding pathways such that...
functional pain continues in the absence of the precipitating cause.

4.2. Sustained stimulation of $\beta_2$- and $\beta_3$ARs leads to neuroinflammation

In previous studies, we demonstrated that acute COMT-dependent pain is initiated by peripheral $\beta_2$- and $\beta_3$ARs through release of the pro-inflammatory cytokines TNF$\alpha$, IL-1$\beta$, and IL-6 in plasma within several hours (Giszerek et al., 2016; Hartung et al., 2014). Here, we found that plasma levels of TNF$\alpha$, IL-1$\beta$, and IL-6 decreased, while CSF levels of these cytokines increased 1–3 weeks following cessation of OR486. This transition from acute inflammation in peripheral tissues to neuroinflammation has been well-characterized within the context of neuropathic, inflammatory, and cancer-related pain (Ji et al., 2016), and may also play a critical role in the chronification of functional pain.

Cytokines are small intracellular regulatory proteins secreted by immune cells in the periphery and neurons and glia in the central nervous system (Kress and Sommer, 2004; Miller et al., 2009; Xanthos and Sandkuhler, 2014). Pro-inflammatory cytokine levels are elevated in patients with FM (Bazzichi et al., 2007; Gur et al., 2002; Sommer et al., 2008; Wallace et al., 2001; Zhang et al., 2008), TMD (Kaneyama et al., 2002; Matsumoto et al., 2006; Ogura et al., 2010; Slade et al., 2011; Takahashi et al., 1998), TTH (Bo et al., 2009; Della Vedova et al., 2013; Kocer et al., 2019), IBS (Dinan et al., 2008; Liebregts et al., 2007; Rana et al., 2012), and pelvic pain (Liebregts et al., 2007; Lindenlaub and Sommer, 2003; Poole et al., 1999), such that higher levels are associated with greater pain (Gur et al., 2002; Kopp, 1998; Ogura et al., 1997; Scanzello, 2017; Shafer et al., 1994; Takahashi et al., 1998). While pro-inflammatory cytokines confer survival advantage in an acute setting by promoting immune responses that limit tissue damage and initiate remodeling (Bennett and Schultz, 1993; Dinarello et al., 1990; Gharaei-Kermani and Phan, 2001), persistent elevations result in tissue pathology and altered nociceptor function. TNF$\alpha$, IL-1$\beta$, and IL-6 increase the activity of nociceptors by direct receptor-mediated actions as well as by inducing the transcription of pain-relevant genes that promote long-term synaptic plasticity (Dansereau et al., 2008; Gao et al., 2009; Ji et al., 2014; Jung et al., 2009; Kawasaki et al., 2008; Millar et al., 2017; Morales and Gereau, 2007; Oh et al., 2001; Sommer and Kress, 2004). Hyperactive nociceptors, in turn, secrete chemokines and other glial modulators from their central terminals in the spinal dorsal horn, leading to the activation of microglia and astrocytes (Huh et al., 2017; Tsuda et al., 2017).

In the present study, we found that systemic delivery of OR486 for 14 days led to $\beta_2$- and $\beta_3$AR-mediated increases in the expression of CD11b, a marker of activated microglia (Ponomarev et al., 2005), and...
GFAP, a marker of activated astrocytes (Venkatesh et al., 2013), in the spinal dorsal horn on day 14 and on day 21, 1 week following OR486 cessation. Microglia and astrocytes are glial cells known to regulate neuroinflammation and pain. Microglia are erythromyeloid-derived glial cells that serve as the resident macrophages of the spinal cord and brain (Kierdorf and Prinz, 2013). They detect adenosine triphosphate, chemokines, and proteases released from sensory afferents and respond by removing debris and resolving damage. Activated microglia promote inflammation and pain through increases in pro-inflammatory cytokines and growth factors that sensitize nociceptive neurons (Ji et al., 2016; Milligan and Watkins, 2009; Yang et al., 2015). Astrocytes are neuroectoderm-derived glial cells that regulate glutamate release and neurotransmission by adjusting concentrations of K+ and Ca++ (De Leo et al., 2006). Similar to microglia, astrocytes release cytokines and chemokines that increase the activity of nociceptive neurons (Ji et al., 2016; Milligan and Watkins, 2009; Yang et al., 2015). Astrocytes are neuroectoderm-derived glial cells that regulate glutamate release and neurotransmission by adjusting concentrations of K+ and Ca++ (De Leo et al., 2006). Similar to microglia, astrocytes release cytokines and chemokines that increase the activity of nociceptive neurons (Ji et al., 2016; Milligan and Watkins, 2009; Yang et al., 2015). Injection of TNFα-activated astrocytes in the spinal cord of naïve mice results in mechanical allodynia that persists for days, thus demonstrating a direct causal role for astrocytes in persistent pain (Gao et al., 2010).

While the exact mechanisms through which microglia and astrocytes contribute to functional pain remain unknown, our data demonstrate that activation of spinal glia precedes increases in CSF pro-inflammatory cytokines. It is possible that the expression of TNFα and other pro-inflammatory cytokines was increased in spinal neurons and glial cells on days 14 and 21, with detection in CSF delayed until day 35. It is also possible that spinal microglia and astrocytes were activated through the release of pro-nociceptive molecules, such as ATP and reactive oxygen species (Basbaum et al., 2009; Sofroniew and Vinters, 2010). Following their stimulation, glia may promote pain transmission via release of inflammatory molecules (eg, cytokines and chemokines) as well as through release of gliotransmitters (eg, glutamate and adenosine triphosphate) that strengthen the responses of first and second order nociceptive neurons (Halassa et al., 2007).

4.3. Sustained stimulation of β2- and β3ARs leads to activation of MAPKs in nociceptors

MAPKs, including p38 and ERK, are intracellular signaling mediators expressed by nociceptors and glial cells that contribute to persistent pain and neuroinflammation following nerve injury or inflammation (Borges et al., 2015; Crown, 2012; Gao and Ji, 2008; Ji et al., 2009), and may also play a role in the chronification of functional pain. We found that systemic delivery of OR486 for 14 days led to β2- and β3AR-mediated increases in neuronal p-p38 expression that remained high on day 21, 1 week following the cessation of OR486. In addition, systemic delivery of OR486 for 14 days led to β2- and β3AR-mediated increases in evoked pERK expression, predominantly in the superficial lamina of
Fig. 6. COMT-dependent functional pain is reversed by intrathecal TNFα or p38 inhibitors, but not by systemic β2- and β3AR antagonists. Animals receiving OR486 for 14 days exhibit mechanical allodynia and hyperalgesia over the course of 35 days. Systemic delivery of ICI118551 + SR59230A for 14 days beginning on day 7 does not reverse OR486-induced (A) mechanical allodynia or (B) mechanical hyperalgesia. Vehicle/Vehicle n = 8; OR486/Vehicle n = 7; OR486/ICI + SR n = 6 males per group. Intrathecal administration of the TNFα inhibitor Etanercept (C, D) or the p38 inhibitor SB203580 (E, F) for 14 days beginning on day 14 reverses OR486-induced mechanical pain. Insets represent average pain behavior following inhibitor delivery on days 21–35. N = 6 males per group. Data are mean ± SEM. **P < 0.01, *P < 0.05 vs. vehicle/vehicle.
The p38 and ERK MAPKs are activated by β2- and β3ARs, through Gi-dependent (Daaka et al., 1997; Schmitt and Stork, 2000; Soeder et al., 1999) and G protein-independent (Aley et al., 2001; Azzi et al., 2003; Cao et al., 2000) mechanisms, as well as by pro-inflammatory cytokines and other nociceptive mediators released from cells on which β2- and β3ARs reside (Ji et al., 2009; Takahashi et al., 2006; Willemen et al., 2010). Upon activation, p38 and ERK stimulate the transcription of pain-relevant genes and modify target proteins to drive peripheral and central sensitization (Chi et al., 2004; Grace et al., 2014; Ji et al., 2009; Koj, 1996; McMahon et al., 2005; Obata and Noguchi, 2004; Seger and Krebs, 1995). For example, inflammatory mediators, injury, and cellular stress activate p38 in DRG neurons, spinal neurons, and spinal microglia (Crown et al., 2008; Jin et al., 2003; Kumar et al., 2003; Moon et al., 2014; Takahashi et al., 2006; Willemen et al., 2010). Activated p38 then translocates to the nucleus and phosphorylates transcription factors (eg, cAMP response element-binding protein; CREB) to stimulate transcription of pro-inflammatory cytokines such as TNFα (Kumar et al., 2003). Activated p38 in spinal interneurons has also been shown to mediate TNFα-induced impairments in the inhibition of pain signals. Similar to p38, activation of ERK following inflammation or injury (Cheng et al., 2008; Gao and Ji, 2010; Ji et al., 2002; Ji et al., 2009; Song et al., 2005; Takahashi et al., 2006; Wei et al., 2006) results in upregulation of CREB-mediated transcription (Song et al., 2005) and glial production of pro-inflammatory molecules (Ji et al., 2009). In contrast to p38, ERK activation is more dynamic, requiring external stimulation (eg, mechanical pinch), and more specific, so that it is restricted to neurons in the superficial dorsal horn (Gao and Ji, 2009). pERK is also considered a marker of central sensitization, as expression levels are correlated with pain progression and chronicity (Ji et al., 2009; McMahon et al., 2005). Inhibition of either p38 or ERK is able to reduce cytokine production, glial activation, and pain (Ji et al., 2009; McMahon et al., 2005).

In line with these investigations, our data show that sustained administration of OR486 over a 14 day period leads to β2- and β3AR-mediated increases in p-p38 and pERK in spinal cord neurons, coincident with the activation of microglia and astrocytes in spinal cord and increased levels of pro-inflammatory cytokines in CSF. Thus, p-p38 and pERK represent downstream effectors of βARs that may contribute to the maintenance of persistent functional pain.

4.4. Contribution of βARs, TNFα, and MAPKs to the maintenance of functional pain

This study demonstrates that sustained stimulation of β2- and β3ARs produces functional pain that persists after removal of the causal stimulus, as well as corresponding increases in spinal pro-inflammatory cytokine production, glial activation, and MAPK phosphorylation. To determine the role of β2- and β3ARs and their downstream effectors (eg, TNFα and MAPKs) in the maintenance of functional pain, we evaluated the ability of specific inhibitors to alleviate pain after it had been established.

While co-administration of the β2AR antagonist ICI118551 and the β3AR antagonist SR59230A blocked COMT-dependent pain and neuroinflammation when delivered on day 0, they did not reverse the pain when delivered on day 7. This finding suggests that peripheral β2- and β3ARs are required for the development, but not the maintenance of functional pain. Consistent with the behavioral results, co-administration of β2- and β3AR antagonists failed to alter COMT-dependent glial activation and MAPKs phosphorylation. Thus, downstream effectors that regulate central sensitization may be more critical for the maintenance of functional pain.

Indeed, we found that intrathecal administration of either the TNFα inhibitor Etanercept or the p38 inhibitor SB203580 on day 14 reversed COMT-dependent mechanical allodynia and hyperalgesia. Thus, TNFα, a key regulator of immune function, and p-p38, a stably expressed MAPK, are essential for the maintenance of functional pain. Etanercept-mediated analgesia on days 21–35 was likely due to inhibition of TNFα in spinal neurons and/or glial cells, as TNFα in CSF was not elevated until day 35. In contrast to TNFα and p38 inhibitors, intrathecal administration of the ERK inhibitor U0126 had no effect on mechanical hypersensitivity. The minimal contribution of pERK to functional pain may be due to the transient nature of its activation only in response to external stimuli.

While Etanercept and other TNFα antibodies have not been evaluated for the treatment of FPS, these anti-TNFα agents are used clinically for the treatment of inflammatory pain conditions, such as arthritis and inflammatory bowel disease (Li et al., 2017), and neuropathic pain conditions, such as lumbar disk hernia and sciatica (Freeman et al., 2013; Tobinick, 2009). However, 40% of patients do not respond to TNFα antibodies (Li et al., 2017). Among responders, long-term administration is associated with increased risk of infection and other adverse events related to off-target activity and antibody-induced immune sensitization (Curtis et al., 2014; Dixon et al., 2006; Feyen et al., 2008; Kroesen et al., 2003; Li et al., 2017). Thus, pharmacologic agents that selectively target intracellular elements within the TNFα signaling network may produce superior therapeutic outcomes for patients with chronic pain in the absence of side-effects associated with long-term antibody regimens.

In line with findings from studies of inflammatory and neuropathic pain (Latremoliere and Woolf, 2009), our results suggest that functional pain is maintained in the central nervous system long after the precipitating cause is removed. Sustained peripheral inflammation can produce neuropaesthetic changes in the spinal cord and brain, so as to increase central responses to peripheral stimuli. This phenomenon may explain why patients with FPS experience sensory abnormalities at regions remote from the original painful site.

5. Conclusions

Collectively, these findings illustrate the importance of β2- and β3ARs in driving neuroimmune signaling events that may underlie functional pain. The onset of COMT-dependent functional pain requires coincident activation of peripheral β2- and β3ARs, while the maintenance requires spinal TNFα and p-p38. Treatments that reduce neuroinflammation linked to abnormalities in catecholaminergic tone (eg, anti-TNFα agents) may prove useful in the management of FPS.

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7. Conflicts of interest

The authors declare no competing interests.

8. Data statement

The authors of this manuscript will provide behavioral and immunohistochemical data upon request.

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