Activation of GPR183 by 7α,25-dihydroxycholesterol induces behavioral hypersensitivity through MAPK and NF-κB

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Running Title: Mechanism of 7α,25-OHC-induced behavioral hypersensitivity

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Abstract:

Emerging evidence implicates the G-protein coupled receptor (GPCR), GPR183 in the development of neuropathic pain. Further investigation of the signaling pathways downstream of GPR183 is needed to support the development of GPR183 antagonists as analgesics. In rodents, intrathecal (i.th.) injection of its ligand, 7α,25-dihydroxycholesterol (7α,25-OHC), causes time-dependent development of mechano- and cold- allodynia (behavioral hypersensitivity). These effects are blocked by the selective small molecule GPR183 antagonist, SAE-14. However, the molecular mechanisms engaged downstream of GPR183 in the spinal cord are not known. Here, we show that 7α,25-OHC-induced behavioral hypersensitivity is Goi dependent, but not β-arrestin 2-dependent. Non-biased transcriptomic analyses of dorsal-horn spinal cord (DH-SC) tissues harvested at the time of peak hypersensitivity implicate potential contributions of mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB). In support, we found that the development of 7α,25-OHC/GPR183 induced mechano-alloodynia was associated with significant activation of MAPKs (extracellular signal-regulated kinase [ERK], p38) and redox-sensitive transcription factors (NF-κB) and increased formation of inflammatory and neuroexcitatory cytokines. SAE-14 blocked these effects and behavioral hypersensitivity. Our findings provide novel mechanistic insight into how GPR183 signaling in the spinal cord produces hypersensitivity through MAPK and NF-κB activation.
**Significance Statement:**

Using a multi-disciplinary approach, we have characterized the molecular mechanisms underpinning 7α,25-OHC/GPR183-induced hypersensitivity in mice. Intrathecal injections of the GPR183 agonist, 7α,25-OHC induce behavioral hypersensitivity and these effects are blocked by the selective GPR183 antagonist, SAE-14. We found that 7α,25-OHC-induced allodynia is dependent on MAPK and NF-κB signaling pathways and results in an increase in pro-inflammatory cytokine expression. This study provides a first insight into how GPR183 signaling in the spinal cord is pronociceptive.
Introduction:

Neuropathic pain is a debilitating disorder that is difficult to treat; and there is an urgent need for novel non-opioid-based analgesics (Finnerup et al., 2015; Yaksh et al., 2018). G-protein coupled receptors (GPCRs) represent the largest class of drug targets in the human genome (Wacker et al., 2017). We have identified a novel role for the GPCR, GPR183 in neuropathic pain (Braden et al., 2020), suggesting it may be a novel analgesic target. GPR183 is most abundantly expressed in lymphoid organs (Rosenkilde et al., 2006), but has been shown to be expressed on astrocytes and microglia in the central nervous system (CNS) of rodents and humans (Rutkowska et al., 2015; Hsiao et al., 2021; Velasco-Estevez et al., 2021). We found that Gpr183 is also expressed in neurons (Braden et al., 2020). The knockout of Gpr183 in mice does not impair fertility and results in a normal gross phenotype with normal numbers of lymphocytes and splenic development (Pereira et al., 2009; Gatto et al., 2013). The endogenous ligand for GPR183 is an oxysterol, 7α,25-dihydroxycholesterol (7α,25-OHC) (Hannedouche et al., 2011; Liu et al., 2011). This oxysterol is a metabolite of cholesterol and is formed by subsequent hydroxylation by the enzymes, cholesterol 25-hydroxylase (CH25H) and 25-hydroxycholesterol 7α-hydroxylase (CYP7B1). 7α,25-OHC is degraded by hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 (HSD3B7) to be further metabolized into bile acids (Mutemberezi et al., 2016).

GPR183 has been implicated in neuroinflammatory and autoimmune conditions such as multiple sclerosis, (Rutkowska et al., 2017; Wanke et al., 2017; Rutkowska et al., 2018; Klejbor et al., 2021) colitis (Misselwitz et al., 2021), type 1 diabetes (Heinig et al., 2010),
and arthritis (Nevius et al., 2015). We have recently reported that traumatic nerve injury engages 7α,25-OHC/GPR183 signaling in the spinal cord that drives the maintenance of central sensitization (Braden et al., 2020). Moreover, intrathecal injection of 7α,25-OHC in mice recapitulates behavioral phenotypes of neuropathic pain and causes a time-dependent development of mechanical and cold-allodynia (behavioral hypersensitivity) (Braden et al., 2020). The molecular mechanism(s) whereby 7α,25-OHC/GPR183 causes hypersensitivities is unknown. Many studies have implicated the roles of neuroinflammation in central sensitization (Matsuda et al., 2019) and previous studies show that at the cell signaling level, 7α,25-OHC-induced activation of GPR183 inhibits adenylate cyclase (AC) activity through Gαi and induces mitogen-activated protein kinases (MAPKs) activation (specifically, extracellular signal-regulated kinase (ERK) and p38) (Benned-Jensen et al., 2011; Rutkowska et al., 2016). MAPKs and other redox-sensitive transcription factors such as NF-κB are crucial to persistent pain sensitization (Ji et al., 2009; Jiang et al., 2020). Using a multidisciplinary approach, this study explores the contribution of spinal MAPKs and NF-κB pathways in response to 7α,25-OHC/GPR183 activation.
Materials and Methods:

Experimental Animals:

Male and female ICR mice (8-12 weeks old; 25-40g starting weight) were purchased from Envigo-Harlan Laboratories (Indianapolis, IN; Milan, Italy). Male C57BL/6J and Arrb2<sup>tm1Rjl</sup>/J (8-9 weeks old; 20-30g starting weight) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed five to ten per cage in a controlled environment (12-hour light/dark cycle) with food and water available ad libitum. All experiments were performed with experimenters blinded to treatment conditions and mice were randomly assigned to treatment groups. All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain, the National Institutes of Health, the Directive 2010/63/EU of the European Parliament, and the ARRIVE guidelines. All studies were approved by the Saint Louis University Animal Care and Use Committee, the University of Messina Review Board, and the Italian Ministry of Health (authorization number 368/2019-PR). Experiments were performed in both male and female rodents; similar results were obtained in both sexes, so data was combined unless noted. No exclusion criteria were set, and no animals were excluded from data, a total of 86 mice were used for these studies.

Test Compounds:

7α,25-dihydroxycholesterol was purchased from Avanti Polar Lipids (Alabaster, AL) and dissolved in DMSO as a 2mM stock. For intrathecal injections 7α,25-OHC was diluted in artificial cerebrospinal fluid (aCSF) (EcoCyte Bioscience; Austin, TX). SAE-14 (Product ID: Z966709080) was purchased from Enamine (Monmouth Jct., NJ), and dissolved in DMSO as a 100mM stock. For intrathecal injections, SAE-14 was diluted in aCSF.
Pertussis Toxin and SN50 were purchased from Sigma-Aldrich (St. Louis, MO). U0126 was purchased from MedChemExpress (Monmouth Jct., NJ). SB202190 was purchased from MedKoo Biosciences (Morrisville, NC). Acute intrathecal (i.th.) injections of compounds were performed as described previously (Lu and Schmidtko, 2013), briefly animals were lightly anesthetized under 2% isoflurane/O2 and the back was shaved. The L5 and L6 spinal processes were identified by aligning the iliac crest and midline of the spinal column. Injections were made between the L5 and L6 vertebrae with a Hamilton syringe attached to a 30-gauge needle. Entrance of the needle into the spinal column was confirmed by a reflexive tail flick prior to injection of compounds; all compounds were administered in a total volume of 5μL. A maximum of two acute intrathecal injections were performed in each mouse.

**Behavioral Testing:**

Mechano-allodynia was measured prior to treatment and at indicated time points, as previously described (Yosten et al., 2020). Briefly, mice were acclimated to a glass boxes on a wire mesh platform for at least 30 minutes before calibrated von Frey filaments (Stoelting; ranging from 3.22 (0.16 g) to 4.31(2.00 g) bending force) were applied to the plantar surface of the hind paw according to the up-and-down method (Dixon, 1991). Paw withdrawal threshold (PWT) was defined as the median 50% threshold force in grams needed to elicit a withdrawal response, calculated according to Chaplan and colleagues (Chaplan et al., 1994). Data were converted to log10 PWT in milligrams prior to statistical analyses as previously described (Sant'Anna et al., 2016). Mechano-allodynia was defined as a significant (p<0.05) reduction in mechanical PWT (g) compared to baseline forces (before treatment).
RNA-sequencing (RNA-Seq):

Male ICR mice were treated with either intrathecal Vehicle or SAE-14 (GPR183 antagonist; 5.2ng/5µL) 30 minutes prior to either intrathecal Vehicle or 7α,25-OHC (GPR183 agonist; 1ng/5µL). Two hours after the last intrathecal injections mice were deeply anesthetized using ketamine/xylazine cocktail and underwent transcardiac perfusion with 1X phosphate buffered saline (PBS) and lower lumbar dorsal horn spinal cord was harvested and placed in RNALater (Sigma Aldrich, St. Louis, MO). Total RNA was extracted from DH-SC of mice using RNeasy Plus Universal Mini kit (Qiagen, Germantown, MD). RNA was then reverse transcribed into cDNA, followed by the construction and sequencing of the cDNA library following the manufacturer’s instructions. RNA-Seq was performed using the Ion Proton deep sequencer (ThermoFisher).

RNA-Seq Data analyses:

Raw RNA-Seq reads were demultiplexed, trimmed to remove adaptor sequences, and converted to the FASTQ format using the Ion Torrent software. The resulting FASTQ reads were pseudo-aligned to the mm10 mouse transcriptome index and counted using the Kallisto software (Bray et al., 2016). Differential gene expression analysis was performed using the edgeR software (Robinson et al., 2010). Gene-level expression was quantified as tags per million reads (TPMs) and TMM-normalized using the edgeR software.

Gene Ontology and Pathway analysis:
Gene list enrichment analysis was performed using the Toppgene Server. Gene set enrichment analysis (GSEA) using pre-ranked genes (Subramanian et al., 2005) was performed using the fgsea R package (Sergushichev, 2016) along with the MSigDB (Molecular Signatures Database) gene sets (V7.4 ) (Liberzon et al., 2015).

Transcription factor analyses of RNA-Seq data:

Overlapping differentially expressed genes between group comparisons were analyzed by ChIP-X Enrichment Analyses (CHEA3) (Keenan et al., 2019) for transcription site enrichment. Only named genes were subjected to enrichment as per program protocol. Gene lists from the analyses were generated by searching for transcription factor subunits commonly associated in the literature with NFκB (Christian et al., 2016), ERK (Le Gallou et al., 2012; Lavoie et al., 2020), and p38 (Asih et al., 2020; Canovas and Nebreda, 2021) signaling.

Western Blot:

Nuclear and cytosolic extracts were prepared as previously described (Campolo et al., 2019). Sample tissues were suspended in extraction buffer A (phenylmethylsulfonyl fluoride (PMSF) 0.2 mM, pepstatin A 0.15 mM, leupeptin 20 mM, sodium orthovanadate 1 mM) and were homogenized and then centrifuged at 12,000 rpm for 4 minutes at 4°C. Supernatants representing the cytosolic fraction were removed and saved for downstream analysis. The pellets, containing enriched nuclei, were resuspended in buffer B (1% Triton X-100, NaCl 150 mM, Tris–HCl pH 7.4 10 mM, EGTA 1 mM, EDTA 1 mM, PMSF 0.2 mM, leupeptin 20 mM, and sodium orthovanadate 0.2 mM) and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatants representing the
nuclear fraction were used for downstream analysis. Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein for each sample were separated on 12% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) non-fat desiccated milk in 1X PBS for 40 minutes at room temperature. Membranes were probed with the following primary antibodies: anti-NF-κB p65 (1:500; Santa Cruz Biotechnology, sc-8008, Dallas, TX); anti-pERK (1:500; Santa Cruz Biotechnology, sc-7383, Dallas, TX), anti-pP38 (1:500; Cell Signalling; cod: 92115; Danvers, MA), and anti-IκBα (1:500; Santa Cruz Biotechnology, sc-1643, Dallas, TX). Secondary peroxidase conjugated goat anti-rabbit IgG or peroxidase conjugated bovine anti-mouse IgG antibodies (1:2000, Jackson ImmunoResearch, West Grove, PA) were used. The following loading control antibodies were used: mouse monoclonal lamin A/C antibody (1:5000; Santa Cruz Biotechnology, Dallas, TX) for nuclear proteins; ERK1/2 (1:5000; Santa Cruz Biotechnology, sc-1647, Dallas, TX) for p-ERK; P38 (1:5000; Santa Cruz Biotechnology, sc-81621, Dallas, TX) for p-P38; or β-actin (1:5000; Santa Cruz Biotechnology, sc-8432, Dallas, TX, USA) for IκBα. Signals were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Protein bands were imaged by densitometric scanning of the X-ray films utilizing a GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and relative expression was quantified using Image J, and standardized to ERK1/2, p38, β-actin and Lamin A/C levels.

Enzyme-linked immunosorbent assay (ELISA):

Enzyme-linked immunosorbent assay (ELISA):
Enzyme-linked immunosorbent assay (ELISA) kits were performed to evaluate the concentration of interleukin-1 beta (IL-1β), tumor necrosis factor (TNF), interleukin-10 (IL-10), and interferon beta (IFN-β) according to the manufacturer’s instructions. In brief, samples were thawed on ice and homogenized in specific lysis buffer and centrifuged. Supernatants were collected and stored at -20°C until the measurement. IL-1β, TNF, IL-10, and IFN-β were measured using a microplate reader at 450 nm.

The following kits, for mouse protein identifications, were used: mouse IL-1 β ELISA kit (ab197742, Abcam, Cambridge, UK), mouse TNF α ELISA kit (MBS825075, MyBioSource, San Diego, CA), mouse IL-10 Instant ELISA kit (BMS614INST, Invitrogen, ThermoFisher, Waltham, MA) and mouse IFN- β ELISA kit (MBS2701516, MyBioSource, San Diego, CA).

Immunofluorescence:

Two hours after the last intrathecal injections mice were deeply anesthetized using ketamine/xylazine cocktail and underwent transcardiac perfusion with 1X PBS and 4% paraformaldehyde (PFA) and lower lumbar spinal cord was harvested and post fixed in 4% PFA at 4°C overnight. The tissues were placed in 20% sucrose in 1X PBS overnight then 30% sucrose in 1X PBS overnight for cryopreservation. The tissues then were embedded in OCT by liquid nitrogen cooled 2-methyl butane.

Free floating spinal cord section (30 μm) were blocked for 1h at room temperature in blocking buffer (5% donkey serum in 1X PBS + 0.25% Tween-20 + 1% cold water fish gelatin) with donkey F(ab) anti-mouse IgG (H+L) (1:20, # 715-007-003; Jackson Immunoresearch, West Grove, PA). The sections were then washed in 1X PBS for 3
times, 2 minutes each. Primary antibodies to NeuN (1:500; #MAB377, Millipore-Sigma, St. Louis, MO), GFAP (1:1000; #PA5-18598; ThermoFisher Scientific, Waltham, MA), and IBA1 (1:500; # 234009; Synaptic Systems, Goettingen, Germany) in blocking buffer were incubated with sections for 48h at room temperature. Next, sections were washed extensively and stained for 24h at room temperature with a cocktail (1:300 dilution each) of anti-goat Alexa 488 (#705-545-147; Jackson Immunoresearch, West Grove, PA), anti-mouse Alexa 405 (#A48257; ThermoFisher Scientific, Waltham, MA), anti-chicken Alexa 647 (#703-605-155; Jackson Immunoresearch, West Grove, PA) and DAPI (1:2500, ThermoFisher, Waltham, MA). Sections were mounted in Prolong® Gold (#P36930; ThermoFisher, Waltham, MA) and images were captured on a Leica SP8 Laser Confocal microscope with an objective magnification of 40X and NA = 1.30.

Image analysis:

Images were imported to Huygens Professional and analyzed by Huygens Object Analyzer (SVI, Netherlands). Briefly, a region of interest (ROI) was selected to cover the whole lamina I, II, and III of the dorsal horn based on anti-NeuN signals of each animal. Thresholds of anti-GFAP and anti-IBA1 signals were set identical through images for analysis. Immunofluorescence intensity of antibody signals was divided by the corresponding ROI volume and then loaded into Prism (Graphpad) for graphing.

Statistical analysis:

Data are expressed as mean ± SD for n biological replicates representing one animal. Biochemical and log-transformed behavioral data were analyzed by paired or unpaired t-test or one-way or two-way repeated measures ANOVA with Bonferroni’s multiple comparisons. Significant differences were defined as p<0.05. Statistical analyses were
Results:

7α,25-OHC-induced hypersensitivity is Gαi-dependent:

We have previously shown that intrathecal injection of 7α,25-OHC produces mechanical and cold allodynia in naïve mice in a GPR183-dependent manner (Braden et al., 2020). To examine whether this hypersensitivity is dependent on Gαi mechanisms, we pretreated mice with an intrathecal injection of pertussis toxin (PTX), an ADP-ribosylating toxin that inactivates G_{i/o} proteins (Mangmool and Kurose, 2011). Mice pretreated with PTX (0.1µg) 72 hours prior to intrathecal 7α,25-OHC (1ng) did not develop mechanical allodynia, while mice treated with its vehicle developed significant allodynia within 30 minutes after intrathecal administration of 7α,25-OHC (Fig. 1A).

GPR183 is also known to be internalized after activation by 7α,25-OHC via canonical β-arrestin 2-dependent desensitization (Velasco-Estevez et al., 2021). To determine whether this internalization is important for 7α,25-OHC-dependent hypersensitivity, we gave either wild type (WT) or Arrb2 (β-arrestin 2) knockout mice intrathecal injections of 7α,25-OHC. The overall severity and progression of 7α,25-OHC-induced allodynia were similar in the β-arrestin 2 knockout and WT mice (Fig 1B).

Unbiased RNA-Sequencing of DH-SC reveals that GPR183 activation is associated with increased MAPK and NFκB signaling and neuroinflammation.

Initial investigation of potential mechanisms engaged by 7α,25-OHC/GPR183 signaling within the spinal cord was performed using an unbiased RNA-Seq analysis of DH-SC taken from mice at the time point of peak pain after 7α,25-OHC (2hr; Fig. 2A). Differential gene expression was analyzed for significant changes in gene expression
(p≤0.05 and fold change ≥1.25 cut-off levels) due to 7α,25-OHC treatment (Veh + 7α,25-OHC versus Veh + Veh) and for significant changes in gene expression in 7α,25-OHC-treated mice due to SAE-14 treatment (SAE-14 + 7α,25-OHC versus Veh + 7α,25-OHC). Data revealed that 7α,25-OHC treatment significantly changed the expression of 278 genes (108 upregulated and 170 downregulated) when compared to vehicle treated animals (Fig. 2B,C). Fifty-six of these significantly expressed genes were counter-regulated by SAE-14 (16 of the 108 genes that were found to be upregulated and 40 of the 170 genes that were found to be downregulated; Figs. 2B-D).

Gene ontology (GO) and pathway analysis revealed that the 56 genes regulated by 7α,25-OHC/GPR183 signaling are involved in a variety of biological processes (Fig. 2E). Within these processes, we found significant enrichment of functions involving immune responses and Toll-like receptor signaling (Fig. 2D, 2E). We also found enrichment of functions associated with NF-κB and MAPK (ERK and p38) signaling pathways (Fig. 2D, 2E). To further validate the GO enrichment of NF-κB and MAPK signaling, we analyzed our 56 genes using ChIP-X Enrichment Analyses (CHEA3) (Keenan et al., 2019) for potential binding of transcription factors associated with NF-κB, ERK, and p38 signaling. CHEA3 analysis recognized 40 named genes and of these, 45% have the potential for NF-κB binding and 85-88% may be regulated by ERK and/or p38 (Fig. 2F and Supplementary Table 1).

7α,25-OHC-induced hypersensitivity is associated with MAPK and NF-κB.

Our unbiased RNA-Seq findings indicated a role of NF-κB and MAPK signaling in the spinal cord in 7α,25-OHC/GPR183-induced mechano-hypersensitivity. The
development of 7α,25-OHC induced mechano-allodynia was associated with DH-SC upregulation of activated MAPKs (ERK and p38 phosphorylation; Figs. 3A-B & 4A) and NF-κB [reduced IκBα and increased nuclear NF-κB-p65; Figs. 3C & 4B] In order to test whether MAPKs and NF-kB directly contribute to 7α,25-OHC’s effects, we used well-characterized and commercially available inhibitors for each of these pathways. Intrathecal administration of the MAPK/ERK kinase (MEK) 1/2 inhibitor, U0126 (Zhang et al., 2014), the p38 kinase inhibitor, SB202190 (Pavao-de-Souza et al., 2012) or the NF-κB inhibitor SN-50 (Sun et al., 2013) blocked the respective MAPKs and NF-kB activation (Fig 3A-C) and attenuated the 7α,25-OHC induced development of mechanical allodynia (Fig. 3D). These 7α,25-OHC mediated signaling pathways were GPR183-dependent, since SAE-14 blocked the upregulation of activated MAPKs (Fig. 4A) and p65 NF-kB (Fig. 4B). Additionally, 7α,25-OHC induced mechano-allodynia was associated with an increased production of inflammatory and neuroexcitatory cytokines IL-1β and TNF (Fig 4C-D). In contrast, 7α,25-OHC reduced levels of the anti-inflammatory cytokine IL-10 and the Type I Interferon (IFN-I) IFNβ, and this was prevented by SAE-14 (Fig 4E-F) which prevented the associated mechano-allodynia (Fig. 4G) as previously reported (Braden et al., 2020).

**Effects of 7α,25-OHC on spinal glial activation:**

Since our RNA-Sequencing data indicated that intrathecal 7α,25-OHC activated immune response-related pathways and glial cells are known to contribute to nociception (Ji et al., 2013) we were interested in whether glia contribute to GPR183-dependent hypersensitivity. Previous reports show that GPR183 is expressed on microglia and astrocytes (Rutkowska et al., 2015; Velasco-Estevez et al., 2021) and that
GPR183 upregulates on microglia and astrocytes in the DH-SC of rodents following traumatic nerve injury (Braden et al., 2020). Immunofluorescence studies performed in DH-SC harvested at the time of peak 7α,25-OHC- induced allodynia did not show any differences in glial fibrillary acidic protein (GFAP) or ionized calcium binding adaptor molecule 1 (IBA1) immunoreactivity between treatment groups (Fig. 5 A-B).
Discussion:

GPR183 has been shown to exclusively couple to Gαᵢ-proteins (Rosenkilde et al., 2006), and our data using PTX establish that GPR183’s effects on behavioral hypersensitivity are dependent on Gαᵢ signaling. Once activated by 7α,25-OHC, GPR183 also recruits βarrestin 2 and is internalized (Velasco-Estevez et al., 2021). It was recently shown that after short-term stimulation GPR183 is trafficked to late endosomes and the Golgi apparatus before being recycled back to the membrane (Velasco-Estevez et al., 2021). βarrestin 2 is a scaffold protein that regulates the internalization and desensitization of GPCRs but can also mediate G-protein independent signal transduction after GPCR internalization, including MAPK and NF-κB pathways (Ma et al., 2021). GPCR signaling through βarrestins is important in biased signaling and differential effects of agonists (Wootten et al., 2018). In our studies, knockout of βarrestin 2 did not prevent the development of allodynia after i.th. 7α,25-OHC. Together with our PTX data, this indicates that GPR183-dependent hypersensitivity does not rely on βarrestin 2 recruitment, but rather traditional Gαᵢ-protein-dependent signaling. However, it should be noted that in the global βarrestin 2 knockout mice, compensatory βarrestin 1 recruitment may take place. GPR183 activation can recruit βarrestin 1 in zebrafish and in mouse embryonic endothelial cells in vitro (Zhang et al., 2015). Further studies will be necessary to clarify whether βarrestin 1 has any role in 7α,25-OHC induced allodynia. This information will direct future structure activity relationship studies to explore whether compounds that bias GPR183-βarrestin interactions could also be developed as potential analgesics in addition to traditional antagonists.
Our results suggest that GPR183 contributes to the development of 7α,25-OHC-induced behavioral hypersensitivity through activation of MAPK and NF-κB p65 pathways in the spinal cord. The activation of MAPKs and their contribution to 7α,25-OHC-induced hypersensitivity is consistent with previous literature showing that GPR183 signaling activates MAPKs p38 and ERK (Benned-Jensen et al., 2011; Rutkowska et al., 2015) and the importance of ERK and p38 to the development of central sensitization in chronic pain states (Ji et al., 2009; Jiang et al., 2020). However, the involvement of NF-κB signaling in the spinal cord following 7α,25-OHC and attenuation of 7α,25-OHC-induced hypersensitivity by SN50 are peculiar. While NF-κB p65 signaling in the spinal cord is critically important for the development of central sensitization in chronic pain states, previous in vitro studies showed that GPR183 signaling had no effect on NF-κB activation or inhibition (Rosenkilde et al., 2006; Rutkowska et al., 2018; Velasco-Estevez et al., 2021). Thus, SN50 would not be expected to attenuate 7α,25-OHC-induced hypersensitivity. It could be argued that SN50 may be targeting ERK/p38-dependent pathways in our model. SN50 binds to the common nuclear import complex, Rch1 (importin-α)/importin-β heterodimer to disrupt its binding to NF-κB p50 and preventing translocation of NF-κB into the nucleus as well as NFAT, STAT1 and AP-1 translocation (Torgerson et al., 1998). However, additional studies have shown the effects on these latter factors are dose-dependent where SN50 doses at 75 µg/ml block NF-κB, NFAT, STAT1 and AP-1 translocation, but 37.5 µg/ml block selectively block NF-κB translocation (Kolenko et al., 1999). Since our dose of SN50 (0.1 µg/5 µl; 20 µg/ml) was well below doses selective for NF-κB translocation, it is not likely that SN50 directly blocked ERK/p38 signaling. However, it is still possible that GPR183-
induced ERK/p38 signaling that then lead to activation NF-κB p65-dependent pathways and SN50 indirectly blocks GPR183-induced ERK/p38 signaling. Additional studies will be needed to parse out the setup of the signaling network and how it is stimulated by GPR183 signaling.

Our data indicate that GPR183 signaling contributes to proinflammatory cytokine production during 7α,25-OHC-induced behavioral hypersensitivity. IL-1β and TNF are known to cause hyperexcitability of spinal neurons, activate glia, and contribute to both acute and chronic pain states (Matsuda et al., 2019; Vanderwall and Milligan, 2019). Additionally, our studies found that GPR183 activation decreased IL-10 levels in the spinal cord. IL-10 is a key anti-inflammatory cytokine that functions as a check on pro-inflammatory cascades and has been found to be potently antinociceptive (Vanderwall and Milligan, 2019). Through genome-wide association studies GPR183 was identified as a negative regulator of the Interferon regulatory factor 7 (IRF7)-driven inflammatory network (IDIN), which includes IFN-I production (Heinig et al., 2010). Based on this, studies showed that GPR183 limits Toll-like receptor (TLR)-mediated type I IFN production via Gαi (Chiang et al., 2013). These previous reports demonstrate that GPR183 activation can suppress IFN-I levels, which have been shown to reduce neuronal excitability and hypersensitivity in chronic pain models (Wang et al., 2006; Lee et al., 2010; Liu et al., 2016; Liu et al., 2019; Donnelly et al., 2021). In our studies, intrathecal 7α,25-OHC induced a slight but significant decrease in IFNβ levels, and GPR183 antagonism with SAE-14 led to a large increase in spinal IFNβ, further supporting GPR183’s role in suppressing IFN-I responses.
Previous reports including our own have shown that GPR183 is expressed in glia (Rutkowska et al., 2015; Velasco-Estevez et al., 2021), and we found that traumatic nerve injury induces upregulation of GPR183 on microglia and astrocytes, but not neurons (Braden et al., 2020). Therefore, we hypothesized that glial cells may become hyperactive in response to 7α,25-OHC. However, when we stained for the common glial activation markers GFAP (astrocytes) or IBA1 (microglia), we found no difference in expression of either marker or morphological features between groups when tested at 2 hours. This was surprising, as glia have a well-characterized role in the generation and maintenance of chronic pain (Ji et al., 2013). Glia respond to nociceptive signals by increasing the expression of their activation markers (GFAP/S100β, IBA1/Cd11b) and increases in ERK and p38 signaling (Ji et al., 2013). Glia are also the primary source of spinal IL-1β and TNF during pathological pain states (Schäfers et al., 2003; Gajtkó et al., 2020). Although we have found no differences among the various groups at the 2-hour time point after 7α,25-OHC, we cannot completely exclude a role of glia in 7α,25-OHC induced behavioral hypersensitivity as additional time course studies are needed to clarify the role of glia.

This study is the first to investigate the downstream signaling mechanisms engaged by 7α,25-OHC/GPR183 activation in the CNS in the context of central sensitization. This is an important step in characterizing this receptor and validating it as a potential therapeutic target in chronic pain states. Future studies will aim to investigate the roles of GPR183-regulated MAPKs and NF-κB signal transduction mechanisms in chronic pain models.
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**Authorship Contributions:**

*Participated in research design:* Braden, Salvemini

*Conducted experiments:* Braden, Campolo, Chen, Li, Giancotti

*Contributed new reagents or analytic tools:* Esposito, Cuzzocrea, Zhang

*Performed data analysis:* Braden, Campolo, Li, Doyle, Zhang

*Wrote or contributed to the writing of the manuscript:* Braden, Doyle, Zhang, Arnatt, Salvemini
References:


Footnotes *†‡§

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† This work has been presented in part at the following meetings:


‡ Drs. Arnatt and Salvemini have filed US Patent Application 17/080,247, 10/2020 GPR183 Antagonists for the Treatment of Pain for intellectual property generated at Saint Louis University. All other authors claim no conflict of interest.

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Figure Legends:

Figure 1 - 7α,25-OHC-induced hypersensitivity is Ga i-dependent, not βArrestin 2-dependent:

A) In male and female ICR mice (data combined) intrathecal injection of Pertussis Toxin (PTX; 0.1μg/5μL; n=5) but not Vehicle (Veh; 10% glycerol in aCSF; n=6) prevented 7α,25-dihydroxycholesterol (OHC;1ng/5μL)-induced mechanical allodynia. PWT (g) = Paw withdrawal threshold

B) Intrathecal injections of 7α,25-dihydroxycholesterol (OHC; 1ng/5μL) induced mechanical allodynia in male WT (n=4) and Arrb2 knock-out mice (βarr2-KO; n=4). Data are mean ± SD; Two-Way ANOVA with Bonferroni’s multiple comparison; *p<0.05 vs D3 or 0 hours; †p<0.05 vs Veh or WT group for n=4-6/group.
Figure 2 - Unbiased RNA-sequencing of DH-SC reveals that 7α,25-OHC/GPR183 signaling is associated with neuroinflammatory, NF-κB and MAPK signaling pathways. (A) In male ICR mice, an intrathecal injection of 7α,25-OHC (1ng/5μL) led to significant mechano-allodynia when measured at 2 h post injection. Pre-treatment with an intrathecal injection of SAE-14 (5.2ng/5μL) 30 minutes prior to 7α,25-OHC (1ng/5μL) attenuated mechano-allodynia. Vehicles had no effect. PWT (g) = Paw withdrawal threshold. Data are mean ± SD, n=3 per group; Two-Way ANOVA with Bonferroni’s multiple comparison; *p<0.05 vs Veh+Veh; †p<0.05 vs Veh+OHC. (B-F) RNA-Seq analyses of DH-SC harvested from mice in panel A. (B) Venn diagram of the number of significant gene changes (p<=0.05, fold change >=1.25) for 7α,25-OHC-responsive gene expression (Veh + OHC vs. Veh + Veh), SAE-14-responsive gene expression (SAE-14 + OHC vs. Veh + OHC) and those common to both comparisons. (C) The significant (p<=0.05, fold change >=1.25) 7α,25-OHC-dependent log2 fold changes in DH-SC genes from comparisons of vehicle + 7α,25-OHC and vehicle treatment groups and their corresponding changes with SAE-14 treatment. Genes significantly regulated by 7α,25-OHC and SAE-14 treatments are indicated in green (56 genes). P-values in panel C are from paired Wilcoxon signed-rank test using all 108 and 170 genes, respectively. (D) Heatmap of the expression levels (transcripts per million reads; TPMs) and chart indicating gene ontology of the 56 genes regulated by 7α,25-OHC and SAE-14. Gene ontology pathways associated with MAPK, ERK and inflammation are marked in red, genes included in each pathway are marked by a green box. (E) Pathway analysis of the 56 genes differentially regulated by 7α,25-OHC and SAE14. The genes were subject to gene list enrichment analysis using the Toppgene server.
(https://toppgene.cchmc.org/). The p-values were determined using the hypergeometric test. (F). Frequencies of the 56 genes putatively regulated by NFkB, ERK or p38 signaling identified by Chea3 analyses.
Figure 3 – 7α,25-OHC-induced hypersensitivity is associated with activation of ERK, p38, and NF-κB:

Western blot of DH-SC from male ICR mice taken 2 hours post 7α,25-OHC intrathecal injections (1ng/5μL; mice from panel D) show increased A) phosphorylated ERK, B) phosphorylated p38, and C) nuclear NF-κB compared to Vehicle which is attenuated by pretreatment with A) U0126 (2µg/5μL), B) SB202190 (SB; 10µg/5μL), or C) SN50 (0.1µg/5μL) respectively. D) Each pathway inhibitor also prevented 7α,25-OHC-induced mechanical allodynia. PWT (g) = Paw withdrawal threshold. Data are mean ± SD; One-Way or Two-Way ANOVA with Bonferroni’s multiple comparison; *p<0.05 vs 0 hours or Veh group; †p<0.05 vs Veh + OHC group; n=15 per group.
Figure 4 – GPR183 antagonism blocks 7α,25-OHC-induced ERK, p38, and NF-κB signaling:

A) Western blot of DH-SC of male ICR mice harvested at 2 hours post 7α,25-OHC intrathecal injections (1ng/5μL; mice in panel G) reveal 7α,25-OHC induced upregulation of the MAPKs p-ERK and p-p38 which was blocked by SAE-14. B) Western blot for nuclear NF-κB and its inhibitory subunit, IκBα show that 7α,25-OHC induced activation of NF-κB which could be blocked by SAE-14. C-F) ELISA of DH-SC taken 2 hours post 7α,25-OHC intrathecal injections show increased C) IL-1β and D) TNF and decreased E) IL-10 and F) IFN-β after 7α,25-OHC is reversed by SAE-14 pretreatment. In male ICR mice, an intrathecal injection of 7α,25-OHC (1ng/5μL) led to significant mechano-allodynia when measured at 2 h post injection. Intrathecal injection of SAE-14 (5.2ng/5μL) 15 minutes prior to 7α,25-OHC (1ng/5μL) attenuated mechano-allodynia. Vehicles had no effect. PWT (g): Paw withdrawal threshold. Data are mean ± SD, n=5 per group; One-Way ANOVA with Bonferroni’s multiple comparison; *p<0.05 vs Veh+Veh; †p<0.05 vs Veh+OHC.
Figure 5 – Spinal GPR183 signaling has no direct effect on glial activation:

A) Representative images (left panel) showing the antibody signals of GFAP (green), IBA1 (magenta), and NeuN (blue) in DH-SC of male and female ICR mice taken 2 hours after being intrathecally injected with either vehicle or SAE-14 (5.2ng/5µL) 15 minutes prior to being intrathecally injected with 7α,25-dihydroxycholesterol (OHC; 1ng/5µL) or vehicle. Dotted lines represent boundaries of the first three dorsal horn laminae. Scale bar = 100 µm. Zoomed-in images of the white square area in the left panel (100 µm X 100 µm) showing the morphology of astrocytes and microglia represented by antibody signals of GFAP (middle panel) and IBA1 (right panel). B) Quantification of immunofluorescence intensity of GFAP and IBA1 antibody signals in I, II, and III laminae. Data are mean ±SD; One-Way ANOVA with Tukey’s multiple comparison; *p<0.05 vs. Veh group for n=6 mice/group.
Figure 1

A

B

Days post Veh/PTX

Hours post i.th. 7α,25-OHC

Log_{10} PWT (mg)

PWT (g)

Days

Hours

0.5

1

2

3

5

0

1

2

3

5

Hours post i.th. 7α,25-OHC

WT

β-Arrestin 2 KO

OHC

Veh

PTX

*
**Figure 3**

**A**

<table>
<thead>
<tr>
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<th>Veh</th>
<th>OHC</th>
<th>OHC + U0126</th>
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<td>p-ERK</td>
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<tr>
<td>ERK 1/2</td>
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% p-ERK/total ERK1/2

Veh: 50
OHC: 60
OHC + U0126: 40

**B**

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<tr>
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<th>OHC + SB</th>
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<td>p-p38</td>
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<tr>
<td>p38</td>
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% p-p38/total p38

Veh: 20
OHC: 60
OHC + SB: 40

**C**

<table>
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<tr>
<th></th>
<th>Veh</th>
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<th>OHC + SN50</th>
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<tr>
<td>NF-κB</td>
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</tr>
<tr>
<td>Lamin A/C</td>
<td></td>
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</tbody>
</table>

NF-κB: 65 kDa
Lamin A/C: 69 kDa

**D**

Veh + Veh
Veh + OHC
U0126 + OHC
SN50 + OHC
SB202190 + OHC

Log10 PWT (mg)

Veh/ drug
Veh/ OHC

Hours post i.th. 7α,25-OHC

-0.25
0
0.5
1
2

% NF-κB/Lamin A/C

Veh: 20
OHC: 80
OHC + SN50: 60
Figure 4

A

B

C

D

E

F

G
Figure 5

A

GFAP  IBA1  NeuN

Veh

OHC

SAE-14 + OHC

B

GFAP

Immunofluorescence Intensity (per mm$^3$)

Veh  OHC  SAE-14 + OHC

IBA1

Immunofluorescence Intensity (per mm$^3$)

Veh  OHC  SAE-14 + OHC
Activation of GPR183 by 7α,25-dihydroxycholesterol induces behavioral hypersensitivity through MAPK and NF-κB

Kathryn Braden, Michela Campolo, Zhoumou Chen, Ying Li, Luigi Antonio Giancotti, Timothy M. Doyle, Emanuela Esposito, Jinsong Zhang, Salvatore Cuzzocrea, Christopher Kent Arnatt, Daniela Salvemini

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Supplementary Table 1. NF-κB and MAPK controlled genes differentially regulated by 7α,25-OHC and SAE-14.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Upregulated by 7α,25-OHC</th>
<th>Downregulated by 7α,25-OHC</th>
</tr>
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<tbody>
<tr>
<td>NF-κB</td>
<td>Dhfr, Diaph3, Gdpd3, Irak1bp1, Kctd4, Mpeg1, Tlr7</td>
<td>Bcl9l, Cacna1b, Dctpp1, Dok1, Drap1, Pdlim4, Rag1, Snord33, Tapbpl, Thap7, Vgf</td>
</tr>
<tr>
<td>ERK</td>
<td>Cript, Dhfr, Diaph3, Etv1, Iqgap2, Irak1bp1, Kctd4, Nup210l, Sf3b6</td>
<td>Barhl2, Bcl9l, Cacna1b, Ccdc24, Cldn15, Cpa4, Dctpp1, Dok1, Drap1, Dsp, Efcc1, Fam229b, Glis1, Lrriq3, Micall2, Mogat1, Msx2, Pdlim4, Rag1, Snord33, Sp6, Tapbpl, Thap7, Tmc4, Vgf</td>
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<tr>
<td>p38</td>
<td>Cript, Dhfr, Diaph3, Etv1, Iqgap2, Irak1bp1, Kctd4, Mpeg1, Nup210l, Sf3b6, Tlr7</td>
<td>Barhl2, Bcl9l, Cacna1b, Ccdc24, Cpa4, Dctpp1, Dok1, Drap1, Dsp, Efcc1, Fam229b, Glis1, Hoxc12, Lrriq3, Mogat1, Msx2, Pdlim4, Phex, Rag1, Snord33, Sp6, Thap7, Tmc4, Vgf</td>
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