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Mechanisms of G protein-coupled estrogen receptor-mediated spinal nociception

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Abstract

Human and animal studies suggest estrogens are involved in the processing of nociceptive sensory information and analgesic responses in the central nervous system. Rapid pro-nociceptive estrogenic effects have been reported, some of which likely involve G protein-coupled estrogen receptor (GPER) activation. Membrane depolarization, increases in cytosolic calcium and reactive oxygen species (ROS) levels are markers of neuronal activation, underlying pain sensitization in the spinal cord. Using behavioral, electrophysiological and fluorescent imaging studies, we evaluated GPER involvement in spinal nociceptive processing. Intrathecal challenging of mice with the GPER agonist G-1 results in pain-related behaviors. GPER antagonism with G15 reduces the G-1 induced response. Electrophysiological recordings from superficial dorsal horn neurons indicate neuronal membrane depolarization upon G-1 application, which is G15 sensitive. In cultured spinal sensory neurons G-1 increases intracellular calcium concentration and induces mitochondrial and cytosolic ROS accumulation. In the presence of G15, G-1 does not elicit the calcium and ROS responses, confirming specific GPER involvement in this process. Following G-1 intracellular microinjections, cytosolic calcium concentration elevates faster and with higher amplitude compared to extracellular exposure, suggesting subcellular GPER functionality. Thus, GPER activation results in spinal nociception, and the downstream mechanisms involve cytosolic calcium increase, ROS accumulation and neuronal membrane depolarization.

Perspective—Our results suggest that GPER modulates pain processing in spinal sensory neurons via cytosolic calcium increase and ROS accumulation. These findings extend the current

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knowledge on GPER involvement in physiology and disease, providing the first evidence of its pro-nociceptive effects at central levels and characterizing some of the underlying mechanisms.

Keywords

GPR30; estrogen; pain; calcium; ROS

Introduction

In addition to regulating reproductive function, estrogens affect a broad spectrum of neural processes, including learning, memory, emotions and affective state, as well as motor coordination and pain sensitivity⁵⁹. Sex hormone-dependent differences in pain and analgesia have been documented both in human and animal studies, with females presenting more severe, frequent, anatomically diffuse and longer lasting pain than males^{17, 84}. These findings suggest an emerging role for estrogens and androgens in pain processing^{23, 34, 38}. The mechanisms by which estrogens regulate nociception are complex, including direct action on the central and peripheral nervous system, as well as indirect actions via their modulation of the skeletal and immune systems²².

17- β -Estradiol (E2) is the most potent estrogen, known to alter gene expression by activating the nuclear estrogen receptors ER α or ER β , which function as ligand-activated transcription factors^{46, 51, 60, 64}. In addition, E2 can trigger rapid signaling events by activating transmembrane estrogen receptors, such as the G protein-coupled estrogen receptor (GPER, also known as GPR30)^{69, 81}.

Rapid pro-nociceptive effects of E2 have been reported at spinal sites (e.g. in adult male Japanese quail³¹ and peripheral levels (e.g. in male Sprague-Dawley rats)⁴⁴; E2 can also rapidly activate spinal pain pathways⁴⁸. Some of these responses are mediated by GPER^{33, 50, 55}. GPER is expressed on nociceptors and in pain-relevant areas of the rat central nervous system, and the expression levels are similar in the male and female^{28, 42, 50, 55, 77}. GPER activation results in hyperalgesia in male⁵⁰ and female rats⁵⁶. Proposed mechanisms of GPER-mediated hyperalgesia include an increase in cytosolic Ca²⁺³³ or activation of protein kinase C epsilon (PKC ϵ) and phosphorylation of channel transient receptor potential, vanilloid subclass I (TRPV1)^{37, 50} in sensory neurons. Thus, GPER activation at peripheral levels appears to be pro-nociceptive, whereas its involvement in pain processing at the spinal level has not been explored.

Intracellular calcium ions (Ca²⁺) control several neural functions, such as transmitter release, membrane excitability, gene transcription and synaptic plasticity⁵. Previous reports from our group^{4, 10, 41} and others^{33, 69} indicate that GPER activation produces an increase in intracellular Ca²⁺ concentration, [Ca²⁺]_i, in neurons and other cell types. The [Ca²⁺]_i increase is a common signature of pain mediators, leading to the activation of several calcium-dependent kinases (e.g. calcium-calmodulin kinase II α - CamKII α), as well as heme-binding enzymes such as cyclooxygenase-2 (COX-2) and the NO synthase (NOS) family, which are responsible for the production of pain-sensitizing molecules⁵². Moreover, in spinal cord neurons, Ca²⁺ uptake by the mitochondria results in production of reactive oxygen species (ROS)⁴⁹, further released in the cytosol to form peroxynitrite and increase glutamatergic transmission, leading to central nociceptive sensitization⁷².

In the present study we evaluated the GPER contribution to pain processing at the spinal level. We found GPER activation to be excitatory, resulting in depolarization of superficial dorsal horn neurons and nociceptive behavior in mice. The mechanisms of GPER-mediated

nociception involve increases in $[Ca^{2+}]_i$ and spinal ROS accumulation. Part of these results was published in abstract form²⁵.

Materials and methods

Drugs

The GPER agonist G-1⁸ was purchased from Calbiochem, EMD Biosciences (Rockland, MA), morphine was obtained from the National Institute of Drug Abuse and the GPER antagonist G15²⁷ was synthesized and provided by Dr. Jeffrey B. Arterburn. For behavioral studies G-1 and G15 stock solutions of 1 mg/mL in ethanol were diluted to the desired concentration using a vehicle containing 0.9% NaCl, 20% ethanol and 2% Tween 20. Morphine was dissolved in saline (0.9% NaCl) solution. For electrophysiology and imaging studies, G-1 and G15 stock solutions 10⁻²M in DMSO were diluted in artificial cerebrospinal fluid and Hank's balanced salt solution, respectively.

Animals

All animals were purchased from Ace Animal Inc. Boyertown, PA. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Behavioral assay

Experiments were performed on male ICR mice. *In vivo* studies of GPER physiology have been carried out in both male and female animals, suggesting its importance in both sexes. The levels of estrogen activating GPER are in the low nanomolar range (3–7 nM)^{66, 67}, whereas plasma estrogen levels are typically in the picomolar range. However, local estrogen concentrations can be considerably higher³⁶. Estradiol is synthesized from testosterone by the enzyme aromatase in both peripheral organs and central nervous system of males and females⁹. High local estradiol concentrations can be rapidly obtained in the male spinal cord (e.g. in the Japanese quail) due to the large available pool of testosterone; this has been shown to be relevant for estrogenic regulation of nociception^{30, 31}. Moreover, the knockout of aromatase enzyme in the male results in reduced fertility, osteoporosis and increased intra-abdominal adiposity⁶¹, suggesting estrogens can regulate similar processes in the male and female. Given GPER expression is similar in the male and female rodent central nervous system^{28, 42}, we considered male animals appropriate for studying GPER involvement in spinal pain.

Animals weighing 18–23 g, n=6–10/group, were maintained at room temperature (22–25 °C), with free access to tap water and standard food, under a 12:12-h light/dark cycle (lights on at 8:00 h), with constant temperature (22 ± 1 °C) and relative humidity (55 ± 5%) and housed 5 animals/cage. Mice were acclimated to the laboratory for at least two days before testing and used only once throughout experiments. Approximately 60 min before injections, the mice were adapted to an individual box which was also used as the observation chamber after the intrathecal (i.t.) (challenging) injection.

The i.t. injection procedure was according to the method described by Fairbanks³²: a 30-gauge stainless steel needle attached to a 50 µL Hamilton microsyringe was inserted between L5 and L6 of an unanaesthetized mouse and drugs given in a volume of 5 µL over the course of 1–2 s. Puncture of the dura was indicated by a reflexive lateral flick of the tail or the formation of an S shape by the tail. Following the injection, the needle was maintained 15–20 s to ensure total delivery of the drug, then the syringe was rotated slightly and removed.

Each mouse received a subcutaneous (s.c.) injection of either vehicle (0.9% saline with 20% ethanol and 2% Tween 20) or chemical (G-15, morphine), followed by an intrathecal injection of G-1 or vehicle 30 min later. Immediately after the i.t. injection, the mouse was placed in the transparent observation box and the accumulated response time of hind limb scratching directed toward the flank, biting and/or licking of the hind paws and the tail was measured for 10 min.

RT-PCR

Adult male ICR mice weighing 20–25 g were rapidly decapitated and the spinal cord surgically removed; cervical, thoracic, lumbar and sacral segments were identified and sectioned, then all tissues were placed in ice-cold Trizol® reagent and immediately frozen (–70 °C).

Total RNA from various tissues was isolated using Trizol® reagent according to the manufacturer's protocol (Invitrogen). The purity and concentration of RNA was assessed spectrophotometrically. First-strand cDNA was synthesized from 2 µg of total RNA with random hexamer primers and the SuperScript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). Two µL of 20 µL cDNA product were amplified with the following specific primers based on mouse GPER (NM_029771) and β-actin (NM_007393) sequences: GPER: 5'-TCA GCA GTA CGT GAT TGC CCT CTT-3' (upstream), 5'-ACT GCT CTG TGC TGT CTG GAA TGA-3' (downstream); β-actin: 5'-TCG TAC CAC AGG CAT TGT GAT GGA-3' (upstream), 5'-ACT CCT GCT TGC TGA TCC ACA TCT-3' (downstream). Amplifications were performed using Platinum Taq DNA polymerase (Invitrogen) as follows: GPER: 32 cycles of 94 °C for 60s, 57 °C for 60s, and 72 °C for 60s; β-actin: 29 cycles of 94 °C for 30s, 55 °C for 45s and 72 °C for 60s. The PCR products were separated using agarose gel electrophoresis on 1% agarose gels and visualized by an ethidium bromide staining under UV light.

Electrophysiology

Visualized whole-cell patch clamp was performed on spinal cord slices from Sprague-Dawley rats of both sexes 5–12 days old. Animals were rapidly decapitated and the spinal cord removed surgically and placed in an ice-cold high sucrose artificial cerebrospinal fluid (ACSF) containing (in mM): 248 sucrose, 11 glucose, 23 NaHCO₃, 2 KCl, 1.25 KH₂PO₄, 2 CaCl₂, 2 MgSO₄, saturated and continuously bubbled with 95% O₂ – 5% CO₂¹⁶. Transverse slices (250 µm thick), at the lumbar level, were cut using a Vibratome (series 1000), and stored for at least one hour at room temperature (21 ± 0.5 °C) into regular ACSF containing (in mM): 126 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 10 glucose, pH 7.35. One slice was transferred to the recording chamber (RC-22, Harvard Apparatus) and superfused continuously with oxygenated ACSF at a mean flow rate of 3 mL/min, at room temperature.

Patch pipettes were pulled from thin-walled fiber-filled borosilicate glass (WPI, OD 2.0 mm), filled with an intracellular recording solution containing (in mM): 130 K⁺ gluconate, 1 Mg Cl₂, 2 CaCl₂, 4 ATP, 10 EGTA, 10 HEPES, pH 7.3 and had resistances of 3–7 MΩ. Cells were visualized using a Nikon (Eclipse FN1) upright microscope and the ones located in the superficial laminae were approached by the patch electrode and a giga-ohm seal established. Recordings were made using a CV-4 headstage (Molecular Devices) attached to an Axopatch 1D amplifier (Axon Instruments/Molecular Devices). Once the whole-cell configuration was obtained, cell membrane potential was recorded in current-clamp mode (I = 0 pA). Baseline membrane potential was initially recorded to ensure that the cell was stable. Pharmacological compounds were administered by bath superfusion at a rate of 3 mL/min and the data was digitized and analyzed using a Digidata 1320A analog-digital

converter (Axon Instruments/Molecular Devices) and the pClamp 10.2 software (Molecular Devices).

Neuronal culture

Spinal cord neurons were dissociated from neonatal (1–2 day old) Sprague Dawley rats of both sexes as previously described^{11, 14, 28}. In brief, newborn rats were decapitated and the spinal cord quickly removed surgically and immersed in ice-cold Hanks balanced salt solution without calcium and magnesium (Mediatech, Herndon, VA). Tissues were minced and subject to enzymatic digestion with papain for 3–4 min at 37 °C, followed by mechanical trituration in presence of total medium (Neurobasal-A (Invitrogen, Carlsbad, CA) containing 1% GlutaMax (Invitrogen), 2% penicillin-streptomycin-amphotericin B solution (Mediatech, Manassas, VA) and 10% fetal bovine serum) to stop enzymatic activity. After centrifugation at 500g, fractions enriched in neurons were collected and resuspended in total medium. Cells were plated on round 25 mm glass coverslips previously coated with poly-D-lysine (Sigma-Aldrich, St Louis, MO) in 6-well plates. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO₂. The mitotic inhibitor cytosine β -arabino furanoside (1 μ M) (Sigma-Aldrich, St Louis, MO) was added to the culture the third day to inhibit glial cell proliferation⁷³. The media was changed every 2–3 days. Cells were used after 3–5 days in culture.

Calcium imaging

Measurements of $[Ca^{2+}]_i$ were performed as previously described^{14, 15, 26}. Cells were incubated with 5 μ M fura-2 AM (Invitrogen, Carlsbad, CA) in Hanks' balanced salt solution (HBSS) at room temperature for 45 min, in the dark, washed three times with dye-free HBSS, and then incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips (25 mm diameter) were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Optical Apparatus Co., Ardmore, PA). The microscope is equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Roper Scientific, Optical Apparatus Co.). During the experiments the Perfect Focus System was activated. Fura-2 AM fluorescence (emission = 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using NIS-Elements AR 3.1 software (Nikon/Optical Apparatus Co.). The ratio of the fluorescence signals (340/380 nm) was converted to Ca^{2+} concentrations³⁹. Those cultured cells with neurites at least three times longer than the cell bodies, which respond to 20 mM KCl solution by an increase in $[Ca^{2+}]_i$ were considered neurons¹¹. Cells with a small (< 10 μ m) soma diameter were considered sensory neurons; previously published immunohistochemical studies of the rat spinal cord²⁸ showed GPER immunoreactivity in the small (< 10 μ m diameter) superficial dorsal horn neurons, as well as in larger neurons (20–25 μ m diameter) located in the ventral horn.

Intracellular microinjection of cultured spinal sensory neurons

Injections were performed using Femtotips II, InjectMan NI2 and FemtoJet systems (Eppendorf) as previously reported^{12, 15, 26, 83}. Pipettes were back filled with and intracellular solution composed of 110 mM KCl, 10 mM NaCl and 20 mM HEPES (pH 7.2)⁴⁰ or G-1. The injection time was 0.4 s at 60 hPa with a compensation pressure of 20 hPa in order to maintain the microinjected volume to less than 1% of cell volume, as measured by microinjection of a fluorescent compound (Fura-2 free acid)⁴⁰. The intracellular concentration of G-1 was determined based on the G-1 concentration in the pipette and the volume of injection.

Detection of cytosolic ROS accumulation

Assessment of cytosolic ROS levels was achieved using H₂-DCFDA dye (2',7'-dichlorodihydrofluorescein diacetate) (Invitrogen, Carlsbad, CA). This assay is based on the principle that the nonpolar, nonionic H₂-DCFDA crosses the cell membrane and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent H₂-DCF. In the presence of ROS, H₂-DCF is rapidly oxidized to become highly fluorescent DCF. Cells were incubated with 1 μ M H₂-DCFDA in HBSS at room temperature for 15 min, in the dark and washed with dye-free HBSS. Coverslips (25 mm diameter) were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Optical Apparatus Co., Ardmore, PA). The intensity of green fluorescence following excitation at 495 nm was acquired at a frequency of 0.25 Hz and evaluated as a measure of cytosolic ROS accumulation.

Detection of mitochondrial superoxide accumulation

Measurement of mitochondrial ROS levels was achieved using the MitoSOX Red superoxide indicator (Invitrogen, Carlsbad, CA). MitoSOX Red reagent permeates live cells, where it selectively targets the mitochondria. At the mitochondrial level, it is rapidly oxidized by superoxide, but not by other ROS and reactive nitrogen species (RNS); the oxidized product is highly fluorescent upon binding to nucleic acid. Cells were incubated with 3 μ M MitoSOX Red in HBSS at room temperature for 25 min, in the dark and washed with dye-free HBSS. Coverslips (25 mm diameter) were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Optical Apparatus Co., Ardmore, PA). The intensity of red fluorescence after excitation at 510 nm was acquired at a frequency of 0.25 Hz and evaluated as a measure of mitochondrial superoxide accumulation.

Statistical analysis

The data are expressed as the mean $\pm$ standard error of mean (S.E.M.). Statistical analysis of difference between treatment groups was assessed with one-way or two-way analysis of variance (ANOVA) followed by Bonferroni test using Origin 7 (OriginLab Corporation, Northampton, MA). ID₅₀ values for G15 with 95% confidence limits were determined by linear regression analysis using Origin 7 software.

Results

Behavioral response induced by spinal GPER activation

Intrathecal (i.t.) administration of the GPER agonist G-1⁸ to male ICR mice produced a characteristic behavioral response consisting of scratching, biting and licking directed caudally, to the tail and hind limbs, along with a slight hind limb scratching directed toward the flank. The total time the mice engaged in this specific behavior upon G-1 (0.1 nmol, 0.5 nmol, 1 nmol/mouse) treatment was $45 \pm 13.4^* \text{ s/10min}$ ($n = 8$), $60.6 \pm 5.6^* \text{ s/10min}$ ($n = 10$), $127.3 \pm 19^* \text{ s/10min}$ ($n = 7$), respectively; the responses were statistically significant ($F(3,28) = 19.43$, $*P < 0.0001$) for all the doses tested. The vehicle had no effect ($0 \pm 0 \text{ s/10min}$, $n = 7$) (Fig. 1A). This behavior was apparent 3–5 min after i.t. delivery of G-1 and almost disappeared at 8 min after injection. Thus, mice were observed for 10 min after injection in all behavioral experiments. To determine whether the response elicited with G-1 is pain-related, the effect of morphine on G-1-induced behavior was assessed. Morphine pre-treatment (1, 3, and 10 mg/kg, s.c.) completely abolished the effect of GPER activation at all the analgesic doses tested ($1.75 \pm 0.8^{**} \text{ s/10min}$, $n = 8$; $0 \pm 0^{**} \text{ s/10min}$, $n = 7$; $0 \pm 0^{**} \text{ s/10min}$, $n = 7$, respectively; $F(3,28) = 79.61$; $**P < 0.0001$) (Fig. 1B), suggesting the

behavioral response elicited with G-1 is related to nociception (an intermediary dose of G-1 – 0.5 nmol/mouse - was chosen to show the effectiveness of morphine in this assay).

To further verify GPER specificity of the response, we examined the effect of the GPER antagonist G15²⁷ on G-1-induced pain-related behavior. G15 (0.1, 1 and 10 nmol/mouse, s.c.) caused a dose-dependent inhibition (34.8 ± 8 s/10min, $n = 7$; $20 \pm 4.2^*$ s/10min, $n = 6$; $4.5 \pm 2^*$ s/10min, $n = 6$, respectively; $F(3,21) = 10.97$, $*P < 0.001$) of caudally directed biting and scratching produced by i.t. G-1 (0.1 nmol/mouse) with an ID_{50} value of 3.95 ($2.6 - 5.3$) nmol/mouse, (Fig. 1C). We have carried out experiments with G15 and an intermediary dose of G-1 (0.5 nmol/mouse); however, the stable solution with the highest concentration of G15 failed to produce satisfactory inhibition of G-1 0.5 nmol/mouse (data not shown) and we had to use a lower dose of agonist (0.1 nmol/mouse). GPER presence was confirmed in the mouse lumbar spinal cord at the mRNA level (Fig 1D).

G-1 depolarizes spinal superficial dorsal horn neurons

The response of a laminae II spinal neuron is shown in Fig. 2A: G-1 (100 nM) depolarized the neuron from a baseline level of -50.5 mV to -41 mV. The G-1 induced depolarization returned to a baseline level of -50.4 mV following drug washout. Fig. 2B presents a representative recording from a neuron pre-treated with G15, where G-1 failed to induce depolarization of the membrane. G15 application alone ($1 \mu\text{M}$) did not change the average membrane potential from baseline levels ($V_m = -57 \pm 2.4$ mV, $n=15$, Fig.2C). Nevertheless, G15 pre-treatment blocked G-1 induced depolarization ($F(1,32) = 121$, $P < 0.00001$, $n=15$) in all superficial dorsal horn neurons tested (Fig. 2C). G-1 depolarized 61% (19/31) of the superficial dorsal horn neurons from an average baseline of -58.3 ± 1.6 mV to -53 ± 1.7 mV ($n=19$), producing a mean depolarization of 5.3 ± 0.4 mV (Fig. 2C, D). 39% (12/31) of the neurons tested did not change membrane potential upon G-1 treatment ($V_m = -57.8 \pm 1.6$ mV).

GPER activation elevates $[Ca^{2+}]_i$ in cultured spinal sensory neurons

a) Extracellular administration of G-1 increases cytosolic Ca^{2+} in a concentration-dependent manner—Fig 3A depicts Fura-2 AM fluorescence ratio (340/380 nm) before and after administration of G-1 (100 nM) in the absence and presence of G15 ($1 \mu\text{M}$). $[Ca^{2+}]_i$ elevation elicited by G-1 started 1–3 min after G-1 administration and reached a plateau after 5–7 min; representative traces are shown in Fig 3B. The mean basal cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, of cultured spinal sensory neurons was 124 ± 3.2 nM ($n = 151$ cells). Bath application of G-1 (10 nM, 100 nM, 1000 nM) induced a concentration-dependent rise in $[Ca^{2+}]_i$, with a mean amplitude of 114 ± 2.6 nM ($n = 37$ cells), 617 ± 6.1 nM ($n = 43$ cells) and 1040 ± 11.2 nM ($n = 29$ cells), respectively, $F(3,255) = 117095.49$, $P < 0.00001$ (Fig. 3C). Pre-treatment with GPER antagonist G15 ($1 \mu\text{M}$, 15 min) largely abolished (15 ± 1 nM, $n = 42$ cells, $F(1,83) = 80110.05$, $P < 0.00001$) the increase in $[Ca^{2+}]_i$ induced by G-1 (100 nM) (Fig. 3C).

b) Intracellular microinjection of G-1 elevates $[Ca^{2+}]_i$ —Representative changes in Fura-2 fluorescence ratio (340/380 nm) before, during and 6 min after injection of control vehicle, G-1 (100 nM, final concentration inside the cell) and G-1 (100 nM) after pre-treatment with G15 ($1 \mu\text{M}$) are shown in Fig 3 A, B and C, respectively. Intracellular microinjection of G-1 resulted in a robust and transitory increase in $[Ca^{2+}]_i$, by 1167 ± 59 nM ($n = 16$ cells, $F(1,25) = 499.2$, $P < 0.00001$ compared to control), whereas control buffer microinjection elevated $[Ca^{2+}]_i$ only slightly, with a mean amplitude of 34 ± 12 nM ($n = 11$ cells). When compared to extracellular application, the effect of microinjected G-1 was faster (seconds vs. minutes) and presented higher amplitude ($F(1,57) = 450.3$, $P < 0.00001$).

Pre-treatment with G15 (1 μ M, 15 min) significantly diminished (209 ± 26 nM, $F(1,28) = 495.69$, $P < 0.00001$) the effects of intracellular G-1 injection (Fig. 4 D, E) ($n = 14$ cells).

G-1 induces cytosolic and mitochondrial ROS accumulation

Stimulation of H2-DCFDA loaded spinal sensory neurons with G-1 (100 nM) lead to a 3.3 times (231%) increase in relative fluorescence (2495 ± 205 , $n=23$), which is statistically significant ($F(1,39) = 46.2$, $P < 0.00001$) when compared to untreated cells (752 ± 121), suggesting oxidation of the dye in response to cytosolic ROS production (Fig. 5A). G15 (1 μ M) pre-treatment prevented G-1 from inducing cytosolic ROS accumulation, maintaining relative fluorescence at basal levels (643 ± 91 , $n=17$, $F(1,38) = 53.69$, $P < 0.00001$) (Fig. 5A).

Mitochondrial superoxide production was significantly elevated ($F(1,23) = 27.4$, $P < 0.00005$) in spinal sensory neurons upon G-1 (100 nM) incubation as suggested by the 2.1 times (110%) increase of MitoSOX Red fluorescence (142 ± 15 , $n=12$ in G-1 treated vs. 59 ± 4 , $n=13$ in untreated neurons) (Fig. 5B). The G-1 effect was largely diminished after G15 (1 μ M) pre-treatment (78 ± 7 , $n=10$, $F(1,20) = 11.86$, $P < 0.005$) (Fig 5B).

Discussion

Activation of GPER on nociceptors has been evaluated both *in vitro* and *in vivo*, indicating peripheral nociceptive sensitization in response to GPER agonists^{33, 37, 50, 55, 56}. However, involvement of GPER in pain processing at central levels is largely unexplored. The present study was aimed at clarifying whether GPER is implicated in spinal nociception.

First, we evaluated the ability of previously characterized GPER ligands^{8, 27} to affect nociceptive behavior initiated at the spinal level *in vivo*. Our data clearly shows that intrathecal challenging of mice with GPER agonist G-1 results in a behavioral syndrome indicative of nociception, consisting of caudally directed biting, scratching and licking. This type of behavior has been described for several pro-nociceptive molecules, such as nociceptin, spermine, D-cycloserine, substance P, excitatory amino acids or sulfur containing amino acids^{1, 2, 45, 63, 70, 71, 76, 78, 79} and has been accepted as indicative of pain if reduced or fully prevented by systemic, intrathecal or i.c.v. morphine. Accordingly, in our experiments, s.c. delivery of analgesic doses of morphine completely abolished G-1-induced nociceptive response. The GPER antagonist G15 dose-dependently diminished the effect of G-1, confirming the specificity of GPER involvement in nociception at the spinal level.

Activation of GPER *in vivo* has been reported to produce pain sensitization. When injected in the hind paw of male rats, G-1 (0.025–2.5 nmol) decreases mechanical nociceptive threshold, pointing to a hyperalgesic effect of peripheral GPER stimulation⁵⁰. Moreover, 2.5–125 nmol/kg G-1, s.c., increases visceral hypersensitivity to 5-hydroxytryptophan in ovariectomized female rats⁵⁶. Both studies are indicative of peripheral nociceptive sensitization, although it is possible that G-1 crossed the blood-brain barrier to enhance hyperalgesic response via central mechanisms. The lowest proalgesic dose of G-1 evaluated in the present study appears to be 0.1 nmol, while the doses reported by Lu et al.⁵⁶ are considerable higher. In addition, we have reported antidepressant effects of 1 nmol G-1 upon i.p. delivery to mice²⁷, indicating that G-1 has the ability to accumulate and elicit responses centrally. Thus, even low systemic G-1 doses, such as those used by Kuhn et al.⁵⁰, are likely to have produced additional pain sensitization upon reaching central levels.

We and others previously reported expression of GPER in the rat spinal cord^{28, 77}. Since the behavioral experiments were carried out in mice, we needed to confirm GPER presence in the mouse spinal cord. We found GPER mRNA to be enriched in the lumbar spinal segment.

This is particularly important since i.t. injections are performed between L5 and L6 and the nociceptive behavior observed in the current study involves the tail and the hind limbs. To our knowledge, there are no reports regarding the possible differential distribution of GPER mRNA in various segments of the rat or mouse spinal cord. Nevertheless, using in situ hybridization, Takanami et al. (2009) showed very low levels of GPER mRNA in the rat lumbar spinal cord, although the lumbar dorsal root ganglia (DRG) presented very high levels; moreover, upon selective spinal dorsal rhizotomy at the lumbar level, GPER immunoreactivity in the lumbar superficial dorsal horn was significantly reduced on the ipsilateral side, suggesting that GPER protein was synthesized in the DRG and then transported via the dorsal root to the terminals of nociceptive afferents in laminae I and II of the spinal dorsal horn. Thus, in the mouse, it is likely for this phenomenon to occur mostly in the cervical, thoracic and sacral spinal regions, while in the lumbar segment GPER is also synthesized locally.

We sought further insight on the mechanism underlying spinal nociceptive effects in response to GPER activation. Neurons in the superficial laminae of the spinal dorsal horn function as the main output source to supraspinal sites, playing a crucial role in the integration of peripheral nociceptive messages^{6, 24, 35}. Depolarization of superficial dorsal horn neurons is involved in central nociceptive sensitization⁴⁷, while analgesic molecules, such as opioids, are known to hyperpolarize neurons in the laminae II of the spinal cord²⁹. Consistent with a pro-algesic effect of GPER stimulation at the spinal level, we found that bath application of G-1 depolarized 61% of the neurons analyzed, while G15 prevented this response. Membrane properties such as resting membrane potential, membrane resistance and time constant are correlated with the neuronal response patterns^{3, 74, 85}. The depolarizations observed with G-1 were only partial, never large enough to produce a depolarization block. However, current-clamp induced partial depolarization of resting membrane potentials turns a neuron with nociceptive specific (NS) functional profile into a neuron with wide dynamic range (WDR) functional profile⁸⁵. NS neurons have passive membrane properties that would require higher amounts of synaptic current to drive the cells toward spike threshold compared to WDR neurons. A modest change in membrane potential makes a NS neuron respond to a low intensity stimulus that would typically affect only the activity of a WDR cell. Therefore, the partial membrane depolarization elicited by GPER activation in postsynaptic neurons is likely to contribute to the development of hypersensitivity to peripheral stimuli in intact animals after G-1 administration.

The increase in $[Ca^{2+}]_i$ is important for neurotransmitter release^{5, 13} and further transmission of nociceptive information. Moreover, membrane depolarization elicits a rapid $[Ca^{2+}]_i$ rise via voltage-gated Ca^{2+} channels, a mechanism involved in pain transmission in superficial dorsal horn (SDH) neurons⁴³. We found that G-1 both depolarizes and increases $[Ca^{2+}]_i$ in spinal cord neurons, with both effects being subject to GPER blockade. This further supports a pro-nociceptive effect of GPER at the spinal level.

Intracellular administration of G-1 to spinal cord neurons resulted in a faster and larger calcium response compared to extracellular application. The difference in kinetics and amplitude suggests an intracellular localization of functional GPER in cultured spinal neurons. Consistent with this observation, in whole tissue or primary cell culture, expression and functionality of intracellular GPER has been reported for hypothalamic neurons, normal and malignant mammary and ovarian epithelia¹⁹, cells of the rat hippocampal formation⁵⁸ or myometrial cells⁸³, although GPER appears to be membrane-associated in renal epithelia¹⁹. In our experiments, pre-treatment with G15 prevented spinal neurons from responding to extra- or intracellular G-1, confirming the GPER specificity of the calcium response.

The increase in $[Ca^{2+}]_i$ in spinal neurons is followed by downstream mitochondrial Ca^{2+} uptake, with consequent production and release of ROS, resulting in synaptic plasticity underlying chronic pain⁴⁹. Indeed, recent research suggests reactive oxygen and nitrogen species are involved in central sensitization associated with acute and chronic inflammatory and noninflammatory neuropathic pain via modulation of protein kinases, alterations in glutamatergic transmission, neuroinflammation and modulation of ion channels such as TRPV1^{72, 86}. These alterations take place in the periphery, in the spinal cord and at supraspinal sites. At the spinal level, ROS contribute to neuropathic pain by reduction of GABA release⁸⁷, sensitization of WDR neurons in the dorsal horn⁵³ and potentiation of synaptic excitability in spinal superficial laminae⁵⁴. We found that GPER activation results in cytosolic and mitochondrial ROS accumulation, which indicates a potential mechanism for GPER-mediated spinal nociceptive sensitization. However, GPER antagonism with G15 does not decrease ROS basal levels, which is important, as superoxide is known to play a beneficial role in learning and memory⁵⁷.

Estrogenic modulation of pain is a complex and controversial topic. Thus, estrogen has been reported to have both analgesic and pro-nociceptive effects depending on the type of pain disorder and associated psychosocial factors, the animal model of pain, the underlying mechanism and the site of action^{22, 65}. For example, very high estradiol levels inhibit inflammation and thus, inflammatory pain, while lower estrogen concentrations are either ineffective or pro-inflammatory^{18, 75}. Temporomandibular joint disorder pain appears to be either enhanced or decreased by estradiol, depending on the receptor subtype it activates^{22, 80}. However, estrogen increases nociception in animal models of migraine headache and trigeminal pain⁷, and clinical studies advocate the pro-nociceptive effects of estrogens on visceral pain³⁸. Moreover, female rats in high estrogenic states or ovariectomized and supplemented with estradiol fail to present proper analgesic responses to spinally delivered orphanin FQ or clonidine^{20, 21, 62, 82}.

The data presented here illustrate that GPER activation results in spinal nociceptive sensitization via second order neuron depolarization, increased cytosolic calcium concentration and ROS generation. These findings do not provide evidence that GPER can be incriminated for the sex-related differences in pain and analgesia; additional behavioral experiments should be carried out in ovariectomized and intact female animals with controlled estrous stage.

Our results support previous findings indicative of pro-algesic effects of GPER activation, which indicate that GPER mediates some of the sensitizing effects of estrogen on nociception. Accordingly, GPER may represent a new target for pain management⁵⁰, and its specific modulation may prove beneficial in the treatment of visceral and trigeminal pain disorders, which are more prevalent in high estrogenic states^{22, 38}. Moreover, there is an overall agreement between studies evaluating the detrimental proliferative role of GPER in breast, endometrial and ovarian cancers^{67, 68}, conditions highly correlated with pain. Targeting these forms of cancers with GPER antagonists in order to reduce proliferation may also prove beneficial in alleviating the associated pain.

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Fig. 1. Spinal GPER activation results in nociceptive behavior in mice.

(A) Effects of varying doses (0.1–1 nmol/mouse) of GPER agonist G-1 administered i.t. in mice. The duration of caudally directed scratching, biting and licking induced by G-1 was determined over a 10-min period starting immediately after injection. This type of behavior was not observed in vehicle-treated mice. * $P < 0.01$ when compared to vehicle. (B) Effect of morphine on G-1-induced scratching, biting and licking response in mice. Morphine was administered s.c. 30 min prior to i.t. (challenging) injection of G-1. ** $P < 0.0001$ when compared to G-1 alone. (C) Effect of GPER antagonist G15 on G-1-induced nociceptive behavior in mice. G15 was given s.c. 30 min prior to i.t. injection of G-1. * $P < 0.001$ when compared to G-1 alone. A–C, Data are given as the means and S.E.M. for groups of 6–10 mice. (D) RT-PCR results of GPER mRNA in different segments of the mouse spinal cord, C-cervical, T-thoracic, L-lumbar, S-sacral. Housekeeping gene β -actin was used as an internal control. The product size of mouse GPER was 944 bp and that of β -actin was 647 bp.

Fig. 2. GPER stimulation results in depolarization of superficial dorsal horn neurons (SDH) neurons in the rat spinal cord

(A) Representative continuous current-clamp recording of a SDH neuron depolarized by bath-applied GPER agonist G-1 (100 nM). (B) Pre-treatment of SDH neuron with GPER antagonist G15 (1 μ M) prevents G-1-induced depolarization (representative trace). (C) G-1 (100 nM) depolarizes SDH neurons with a medium value of 5.3 mV (n=19). G15 (1 μ M) has no effect on resting membrane potential, but pre-treatment with G15 blocks G-1-induced effect (n=15). *P < 0.00001 when compared to G-1 alone. (D) Depolarizations induced by G-1 (100 nM) in individual SDH neurons (3.11–9.12 mV).

Fig. 3. Extracellular G-1 application elevates cytosolic Ca^{2+} in spinal sensory neurons
(A) Fura-2 AM fluorescence ratio (340/380 nm) before (left) and after (right) administration of G-1 (100 nM) in the absence (top panels) and presence (bottom panels) of G15 (1 μ M); the fluorescence ratio scale (0–2) is magnified in top left panel. **(B)** Representative recordings of cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, in response to different concentrations of G-1 (10–1000 nM) and G-1 (100 nM) after pre-treatment with G15 (1 μ M, 15min). **(C)** Comparison of increases in $[Ca^{2+}]_i$ produced by G-1 (10–1000 nM) and G-1 (100 nM) after pre-treatment with G15 (1 μ M, 15min); G-1 induced a concentration-dependent rise in $[Ca^{2+}]_i$; the effect was abolished by G15. $P < 0.05$ compared to basal $[Ca^{2+}]_i$ (*) or to $[Ca^{2+}]_i$ increase produced by G-1 (100 nM) alone (**).

Fig. 4. Microinjection of G-1 elevates $[Ca^{2+}]_i$ in spinal sensory neurons

A–C, Representative examples of changes in Fura-2 fluorescence ratio (340/380 nm) before, during and 6 min after injection; the fluorescence ratio scale (0–3) is illustrated in each panel and magnified in **A**; the arrows indicate the injected cells. **(A)** Microinjection of control buffer produces a minimal change in fluorescence ratio. **(B)** G-1 (100 nM final concentration inside the cell) microinjection induces a marked increase in fluorescence ratio, which is significantly diminished by G15 (1 mM) pre-treatment **(C)**. **(D)** Representative traces of effects on $[Ca^{2+}]_i$ produced by microinjection of control buffer (black), G-1 (red) and G-1 after G15 pre-treatment (green). **(E)** Comparison of changes in $[Ca^{2+}]_i$ during injection of control buffer, G-1 and G-1 in presence of G15. $P < 0.05$ compared to control buffer (*) or to G-1 alone (**).

Fig. 5. GPER-mediated cytosolic and mitochondrial ROS accumulation in spinal neurons
(A) GPER agonist G-1 (100 nM) increases cytosolic ROS levels in cultured spinal cord neurons, whereas pre-treatment with GPER antagonist G15 (1 μ M) prevents G-1-induced response, maintaining ROS at basal levels. **(B)** G-1 (100 nM) elevates mitochondrial ROS; pre-treatment with G15 blocks G-1 effect. $P < 0.05$ compared to control (*) or to G-1 alone (#).