



Sigma-I receptors and progesterone metabolizing enzymes in nociceptive sensory neurons of the female rat trigeminal ganglia: A neural substrate for the antinociceptive actions of progesterone

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Abstract

Orofacial pain disorders are predominately experienced by women. Progesterone, a major ovarian hormone, is neuroprotective and antinociceptive. We recently reported that progesterone attenuates estrogen-exacerbated orofacial pain behaviors, yet it remains unclear what anatomical substrate underlies progesterone's activity in the trigeminal system. Progesterone has been reported to exert protective effects through actions at intracellular progesterone receptors (iPR), membrane-progesterone receptors (mPR), or sigma I receptors (Sig-IR). Of these, the iPR and Sig-IR have been reported to have a role in pain. Progesterone can also have antinociceptive effects through its metabolite, allopregnanolone. Two enzymes, 5 α -reductase and 3 α -hydroxysteroid dehydrogenase (3 α -HSD), are required for the metabolism of progesterone to allopregnanolone. Both progesterone and allopregnanolone rapidly attenuate pain sensitivity, implicating action of either progesterone at Sig-IR and/or conversion to allopregnanolone which targets GABA_A receptors. In the present study, we investigated whether Sig-I Rs are expressed in nociceptors within the trigeminal ganglia of cycling female rats and whether the two enzymes required for progesterone metabolism to allopregnanolone, 5 α -reductase and 3 α -hydroxysteroid dehydrogenase, are also present. Adult female rats from each stage of the estrous cycle were rapidly decapitated and the trigeminal ganglia collected. Trigeminal ganglia were processed by either fluorescent immunohistochemistry or western blotting to for visualization and quantification of Sig-IR, 5 α -reductase, and 3 α -hydroxysteroid dehydrogenase. Here we report that Sig-IRs and both enzymes involved in progesterone metabolism are highly expressed in a variety of nociceptive sensory neuron populations in the female rat trigeminal ganglia at similar levels across the four stages of the estrous cycle. These data indicate that trigeminal sensory neurons are an anatomical substrate for the reported antinociceptive activity of progesterone via Sig-IR and/or conversion to allopregnanolone.

Keywords

progesterone, trigeminal sensory neurons, allopregnanolone, sigma-I receptor, 5 α -reductase, 3 α -hydroxysteroid dehydrogenase

Introduction

Orofacial pain is a general term used for pain conditions that affect structures of the head, face, neck, and oral cavity. These cranial and orofacial structures are innervated by trigeminal nociceptors that have cell bodies in the trigeminal ganglia and send nociceptive sensory input to the central nervous system. Pain signaled by the trigeminal nociceptors is common, often debilitating,¹ and includes musculoskeletal, neurovascular, and neuropathic pain.^{2,3} In fact, orofacial pain affects 26% of the

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general population with the majority of patients being women.^{4,5} Some orofacial pain disorders, such as temporomandibular joint disorders (TMD),⁶⁻⁸ cervicogenic headaches,⁹ tension headaches,^{10,11} and migraine,¹⁰ are more common prior to menopause, while others, such as trigeminal neuralgia,¹² and burning mouth syndrome,¹³ are more common after menopause. Women also report greater pain sensitivity,¹⁴ less tolerance, and a lower pain threshold than men.¹⁵

Importantly, women report variance in their orofacial pain across their menstrual cycle with pain sensitivity increasing in the late luteal phase and peaking during menses.^{16,17} Women that are pregnant or postmenopausal report a decrease in orofacial pain sensitivity, particularly TMD, with some postmenopausal women reporting their TMD pain reemerges following hormone replacement therapy (HRT).¹⁸⁻²⁰ Preclinical studies also report a reduction in nociceptive responses in pregnant female rats following formalin injection into the temporomandibular joint (TMJ).²¹ Formalin or glutamate injection into the TMJ of female rats also results in increased pain sensitivity during diestrus when 17 β -estradiol levels begin to rise.²² Despite the clear link between fluctuating gonadal hormones and TMD pain, the mechanisms underlying the effects of gonadal hormones on TMD pain remains elusive.

Estrogen and progesterone, the major female gonadal hormones, are primarily synthesized in the ovaries of females. These gonadal hormones are also neurosteroids as they can be locally synthesized in neurons and glial cells,^{23,24} which allows these hormones to directly modulate both the central and peripheral nervous system. Although estrogen has been reported to be both pronociceptive and antinociceptive in various pain models,^{22,25-29} the research literature exclusively reports that progesterone is anti-inflammatory and antinociceptive.³⁰⁻³⁶ We have recently reported that estrogen increases pain behaviors in a rat model of inflammatory TMD pain which is reversed with progesterone treatment.³⁷ Progesterone is also metabolized by 5 α -reductase to 5 α -dihydroprogesterone, which is then converted to the metabolite allopregnanolone by 3 α -hydroxysteroid dehydrogenase (3 α -HSD), thus progesterone's protective effects can occur through its metabolite allopregnanolone. Allopregnanolone is a positive allosteric modulator of the γ -aminobutyric acid type A (GABA_A) receptor, which underlies its antinociceptive properties.³⁸ Indeed, we recently reported that injection of allopregnanolone can attenuate pain behaviors in a rat model of inflammatory TMD pain.³⁷ In support, mechanical and thermal pain thresholds in spinal cord injury animals were reduced following either an intrathecal injection of Provera, a pharmacological inhibitor of 3 α -HSD activity,³⁹ or siRNA knockdown 3 α -HSD.⁴⁰

Despite the evidence that progesterone and allopregnanolone are antinociceptive and attenuate orofacial pain, it is currently unknown whether progesterone or allopregnanolone can act directly at trigeminal sensory neurons. Progesterone can exert protective effects through the intracellular progesterone receptor (iPR), membrane-progesterone

receptors (mPR), sigma-1 receptors (Sig-1R), and its neuroactive metabolite allopregnanolone.⁴¹⁻⁴³ Both iPRs and mPRs are widely expressed within the brain⁴¹ and within the trigeminal ganglia.^{44,45} Although the role of mPRs in nociception is unknown, iPRs alone do not completely attenuate inflammatory TMJ allodynia⁴⁵ indicating other mechanisms, such as allopregnanolone or Sig-1R, at play. Progesterone may be metabolized locally in sensory neurons to allopregnanolone to inhibit pain via GABA_A receptors expressed in trigeminal sensory neurons. Alternatively, progesterone may act at the Sig-1R in trigeminal sensory neurons attenuating nociception. Sig-1R is a non-opioid receptor located within the plasma membrane of the endoplasmic reticulum. Agonists for the Sig-1R elicit nociceptive responses,⁴⁶⁻⁴⁹ which are reversed by antagonists.⁴⁸⁻⁵⁵ Progesterone acts as an antagonist of the Sig-1R.⁴²

The current study was designed to determine whether Sig-1Rs and/or the enzymes involved in the conversion of progesterone to allopregnanolone are present in the nociceptive population of sensory neurons of the female rat trigeminal ganglia. As female rats may have variations in expression levels of membrane proteins when ovarian hormones fluctuate across the estrous cycle,^{56,57} we also examined whether the expression of the enzymes and Sig-1Rs display plasticity in expression levels across diestrus, proestrus, and estrus. Here we utilized fluorescent immunohistochemistry, confocal microscopy, and western blotting techniques to uncover two available mechanisms underlying the effects of progesterone on orofacial pain.

Methods and materials

Subjects

A total of 32 adult female Sprague–Dawley rats (150–200 g; Charles River Laboratories) were used in these experiments. Rats were double-housed in a 12:12 h light-dark cycle with lights on at 8 a.m. Food and water were available *ad libitum*. Rats were acclimated to the facility for 1 week before experiments began. All studies were approved by the Texas Woman's University Institutional Animal Care and Use Committee, conform to federal guidelines, comply with the ARRIVE guidelines, and were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Vaginal cytology

Vaginal lavages were conducted daily between 9 a.m. and 11 a.m. to confirm animals were cycling normally and to determine the phase of estrous cycle on the day of tissue collection. Proestrus was determined by the predominance of nucleated epithelial tissue and estrus was predominantly cornified epithelial tissue. Diestrus 1 was differentiated from diestrus two by the presence of leukocytes. Rats ($n=8$ per

Table 1. Antibodies used for western blotting and fluorescent immunohistochemistry.

Primary antibody	Company	Cat #	Alexa Fluor	Target
Goat anti-SRD5A1	Abcam	AB110123	Donkey anti-goat 647	5 α -reductase
Mouse anti-AKR1C1/IC2	Abcam	AB131375	Goat anti-mouse 568 or donkey anti-mouse 647	3 α -hydroxysteroid dehydrogenase
Rabbit anti-Sigma-1R/OPRS1	Novus Biologicals	NBP1-82479	Donkey anti-rabbit 488 or 555 or goat anti-rabbit 568	Sigma-1 receptor
Guinea pig anti-PGP9.5	Millipore	AB1774	Goat anti-guinea pig 568	Nerve fibers and neurons
Goat anti-Nav1.8	Alomone	SCN10 A	Donkey anti-goat 647	Sodium channel 1.8; nociceptors
Goat anti-CGRP	Abcam	AB36001	Donkey anti-goat 488 or 568	Calcitonin gene-related peptide;
Rabbit anti-CGRP	Immunostar	24,112	Goat anti-rabbit 568	peptidergic neurons
Guinea pig anti-TRPV1	Neuromics	GPI4100	Goat anti-rabbit 488	TRPV1 ion channels; nociceptor subtype
Isolectin IB ₄ Biotin conjugate	Fisher Scientific	I21414	Streptavidin 488 conjugate	Non-peptidergic neurons
Chicken anti-NF200	Abcam	AB4680	Goat anti-chicken 488 or 568	Neurofilament heavy; myelinated neurons

stage) were rapidly decapitated between 9 a.m. and 11 a.m. and their trigeminal ganglia were removed. Tissues collected for western blots were stored at -80°C and tissue for immunofluorescent staining was stored in Tissue-Plus O.C.T (Optimal Cutting Temperature) Compound (SciGen Scientific, Gardenia, CA, USA) at -80°C .

Protein extraction

Total protein extraction of bilateral trigeminal ganglia from 16 rats that were in either proestrus, estrus, diestrus 1, or diestrus 2 ($n = 4$ per stage) were homogenized in lysing matrix tubes (MP Biomedicals; Solon, OH, USA) with PierceTM RIPA buffer (Thermo Scientific; Rockford, IL, USA) with HaltTM protease inhibitor cocktail (Thermo Scientific; Rockford, IL, USA) to prevent proteolysis. Tissue homogenization was performed for 10 s at 6.0 m/s for a total of three cycles, using VWR[®] homogenizer bead mill (Avantor; Radnor, PA, USA). Homogenates were then centrifuged at 13,000 r/min for 15 min at 4°C . Supernatant was collected and protein concentration was determined by PierceTM BCA assay kit (Thermo Fischer Scientific; Waltham, MA, USA). Protein samples were stored until future use at -80°C .

Western blots

Extracted protein was used for protein quantification of 5 α -reductase, 3 α -hydroxysteroid dehydrogenase (3 α -HSD), and sigma-1 receptor (Sig-1R). Equal amounts of protein (20 μg) were loaded into 10% Mini-PROTEAN TGX precast gels (Bio-Rad; Hercules, CA, USA) and run for 100 min at 120 V then transferred to a polyvinylidene fluoride (PVDF) blotting membrane. The membrane was blocked with 5% bovine serum albumin (BSA) in tris-buffered saline (TBS) with 0.01% Tween 20 (TBST) for 1 h at room temperature then

incubated overnight with primary antibody rabbit anti-sigma-1 receptor (Sig-1R; 1:500; Novus Biologicals, NBP1-82,479), goat anti-5 α -reductase (SRD5A; 1:500; Abcam, ab110123), or mouse anti-3 α -hydroxysteroid dehydrogenase (3 α -HSD; 1:500; Abcam, ab131375) at 4°C on a shaker (see Table 1). PVDF membranes were washed three times with TBST then incubated for 1 hour with the corresponding secondary antibody goat anti-mouse (1:8000), donkey anti-goat (1:8000), or goat anti-rabbit (1:8000) at room temperature on a shaker. PVDF membranes were washed three times with TBST and then visualized by Bio-Rad ChemiDocTM MP Imaging System. Protein was quantified by Image J (NIH) and normalized to β -actin or β 3-tubulin. Prostate tissue from adult male rats ($n = 2$) were used as a positive control for 5 α -reductase and tissue from adult male testis ($n=1$) was used as a negative control for 5 α -reductase. The liver was a positive control for 3 α -HSD and Sig-1R. Blocking peptide (NBP1-82,479PEP, Novus Biologicals) was used to confirm specificity of the Sig-1R antibody.

Fluorescent Immunohistochemistry and Confocal Microscopy

A total of 16 rats ($n = 4$ per stage of the estrous cycle) were utilized for immunohistochemistry of Sig-1R, 5 α -reductase, and 3 α -HSD within the trigeminal ganglia. Trigeminal ganglia were cut into 30 μm sections onto slides using a Leica cryostat at -20°C , then stored at -80°C and processed by fluorescent immunohistochemistry within < 12 weeks. Tissue sections were fixed with 4% paraformaldehyde then pre-incubated with filtered normal goat or donkey serum in 0.1 M phosphate buffered saline (PBS) with 0.1% Triton X-100 at room temperature for 90 min. Tissue sections were then incubated with primary antibody (see Table 1) rabbit anti-Sig-1R (1:100; Novus Biologicals, NBP1-82,479), goat anti-SRD5A (1:100; Abcam, ab110123), or mouse anti-3 α -HSD

(1:100; Abcam, ab131375) overnight at room temperature. The tissue sections were then washed with 0.1 M PBS three times and then incubated with the corresponding fluorescent-conjugated secondary antibody goat anti-rabbit Alexa-488 or 568 (1:300; Molecular Probes, Eugen, OR, USA), goat anti-mouse Alexa-488 or 568 (1:300; Molecular Probes), or donkey anti-goat Alexa-647 (1:300; Molecular Probes) for 90 min, rinsed, then subjected to a second round of primary immune-labeling overnight at room temperature with primary antibody for Isolectin IB4 (1:500; Fischer Scientific, I21414) in 0.1 mM CaCl₂, rabbit anti-TRPV1 (1:1000; Alomone, ACC-030), goat anti-Nav1.8 (1:1000; Alomone, SCN10 A), goat anti-CGRP (1:1000; Abcam, AB36001) or rabbit anti-CGRP (1:1000; Immunostar 24,112), guinea pig anti-PGP9.5 (1:1000; Millipore, AB5898), or chicken anti-NF200 (1:1000; Abcam, AB4680).

Tissues were then rinsed with 0.1 M PBS three times for 10 min each, incubated in the dark with fluorescent-conjugated secondary antibody goat anti-rabbit Alexa-568 (1:300; Molecular Probes), donkey anti-goat Alexa-488 or Alex-568 (1:300; Molecular Probes), or goat anti-chicken Alex-488 or Alexa-568 (1:300; Molecular Probes). Tissues incubated with isolectin IB4 primary antibody were first washed two times in 0.1 M PBS then two times in 0.1 mM CaCl₂. For secondary antibody incubation, tissues were incubated with fluorescent-conjugated secondary antibody for streptavidin (1:500; Molecular Probes) for 90 min at room temperature in the dark then slides were washed three times for 10 min with 0.1 M PBS. Slides were then rinsed, dried, and cover slipped using Prolong Gold antifade mountant (P36930; Life Technologies). Mouse IgG2b isotype control (1:100; abcam, ab170192) added to the slide instead of the primary antibody was used as a control for 3 α -HSD to account for any non-specific binding.

Images were acquired with Nikon A1 Confocal Laser Microscope with NIS-Elements C software. All control images were obtained with the same gain settings as experimental images. To account for background signal of the secondary antibody, tissues were stained using the same methods as mentioned above, except the primary antibody was omitted. No non-specific immunoreactivity was observed with secondary antibodies at the concentration used. Representative images were captured across the trigeminal ganglia for cell quantification. Cells expressing the protein of interest (Sig-1R, 5 α -reductase, or 3 α -HSD) and the neuron subpopulation markers (PGP9.5, NF200, IB4, Nav1.8, CGRP, or TRPV1) were manually counted ($n = 2-4$ each) and percent of the protein of interest co-localized within the neuron subpopulation was reported.

Data analysis

Western blots were analyzed by densitometry using Image J software (National Institute of Health, Bethesda, MD). Each blot was selected as a region of interest, sampled 3 times, and

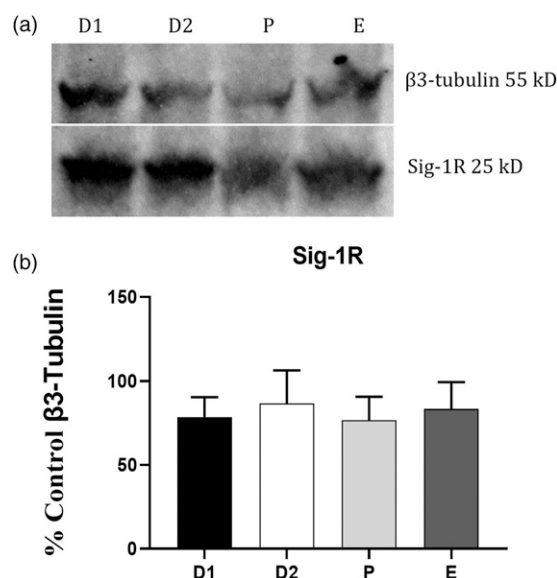


Figure 1. Sigma-1 receptor protein in trigeminal ganglia of the female rat does not vary across the estrous cycle. Sigma-1 receptor (Sig-1R) protein expression detected by western blot (A) and quantified (B) across each phase of estrous cycle [diestrus 1 (D1); diestrus 2 (D2); proestrus (P); estrus (E)] as a percent of beta three tubulin (β 3-tubulin). No significant differences were detected.

analyzed for average gray scale pixel value (sum gray values/number of pixels; 8-bit) following background correction (set at 150 pixels). Data were analyzed by one-way analysis of variance (ANOVA) in Graphpad Prism 8. Tukey's post hoc analysis was conducted. Statistical significance was tested at $p \leq 0.05$.

Results

Sigma-1 receptors are present in nociceptive neural populations of the female rat trigeminal ganglia

Protein levels of Sig-1R expression were analyzed by western blot (Figure 1(a)) across the female rat estrous cycle. Levels of Sig-1R did not significantly vary across the rat estrous cycle [$F(3, 12) = 1.036$; $p > 0.05$] (Figure 1(b)). Sig-1R immunoreactivity (Figure 2(a) and (d)) and PGP9.5 immunoreactivity (Figure 2(b) and (e)) were observed within the same trigeminal ganglia sensory neuron populations (Figure 2(c) and (f)). Sig-1R immunoreactivity (Figure 2(g) and (j)) and NF200 immunoreactivity (Figure 2(h) and (k)) was also observed within the same trigeminal ganglia sensory neuron populations (Figure 2(i) and (l)). All PGP9.5+ cells and NF200+ cells also expressed Sig-1R (open bars; Figure 12(a) and (b)). Sig-1R immunoreactivity was observed in liver (positive control), but not following blocking peptide (negative control) (data not shown). These data reveal that

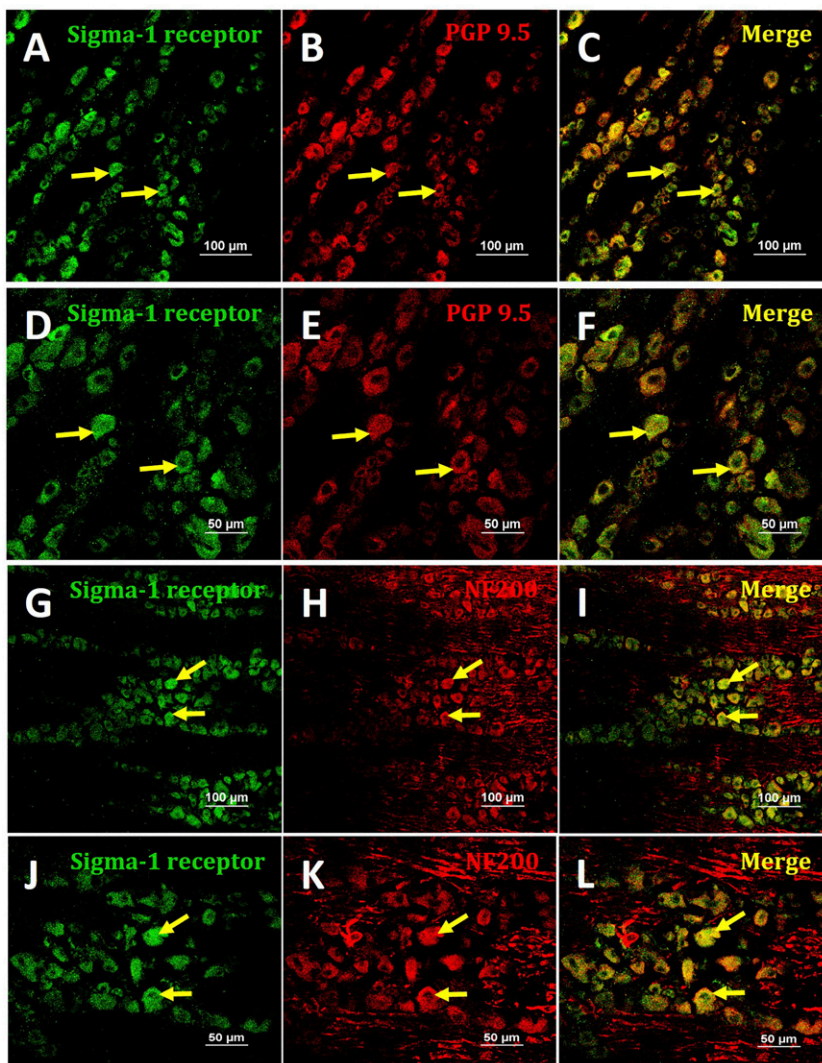


Figure 2. Sigma-1 receptors are expressed on neurons, nerve fibers, and myelinated neurons in the female rat trigeminal ganglia. Immunofluorescent staining of sigma-1 receptor (Sig-1R; green) (A), protein gene product 9.5 (PGP9.5; neurons and nerve fibers; red) (B), and merged image (C) at 20X magnification. Sig-1 R (green) (D), PGP9.5 (red) (E), and merge images (F) at 40X magnification. Immunofluorescent staining of Sig-1R (green) (G), neurofilament heavy (NF200; myelinated neurons; red) (H), and merged image (I) at 20X magnification. Sig-1 R (green) (J), NF200 (K), and merged image (L) at 40X magnification. Arrows indicate cells with protein coexpression.

trigeminal sensory neurons, including myelinated neurons, express Sig-1R.

Sig-1R immunoreactivity (Figure 3(a) and (d)) and Nav1.8 immunoreactivity (Figure 3(b) and (e)) were also observed within the same trigeminal ganglia neurons (Figure 3(c) and (f)). Approximately 79% of the Nav1.8 population coexpressed Sig-1R (open bar; Figure 12(c)). Trigeminal ganglia neurons that were immunoreactive for Sig-1R (Figure 3(h) and (k)) and isolectin IB4 (Figure 3(g) and (j)) were also observed in the female rat trigeminal ganglia in the same neuron populations (Figure 3(i) and (l)). Approximately 69% of the non-peptidergic population expressed Sig-1R (open bars; Figure 12(d)). These data reveal that nociceptive and

non-peptidergic trigeminal sensory neurons largely express Sig-1R.

TRPV1 immunoreactivity was observed in a subpopulation of trigeminal ganglia neurons defined as nociceptive sensory neurons (Figure 4(a) and (e)). TRPV1-positive cells also expressed Sig-1R (Figure 4(b) and (f)), of which about 91% of TRPV1-positive were also positive for Sig-1R (Figure 12(e)). TRPV1 immunoreactivity overlapped with CGRP immunoreactivity, as expected (Figure 4(c) and (g)). The peptidergic TRPV1 population of sensory neurons highly expressed Sig-1R (Figure 4(d) and (h)). In line with the high expression of Sig-1R in the TRPV1-expressing neuron subpopulation, about 92% of the CGRPergic population also

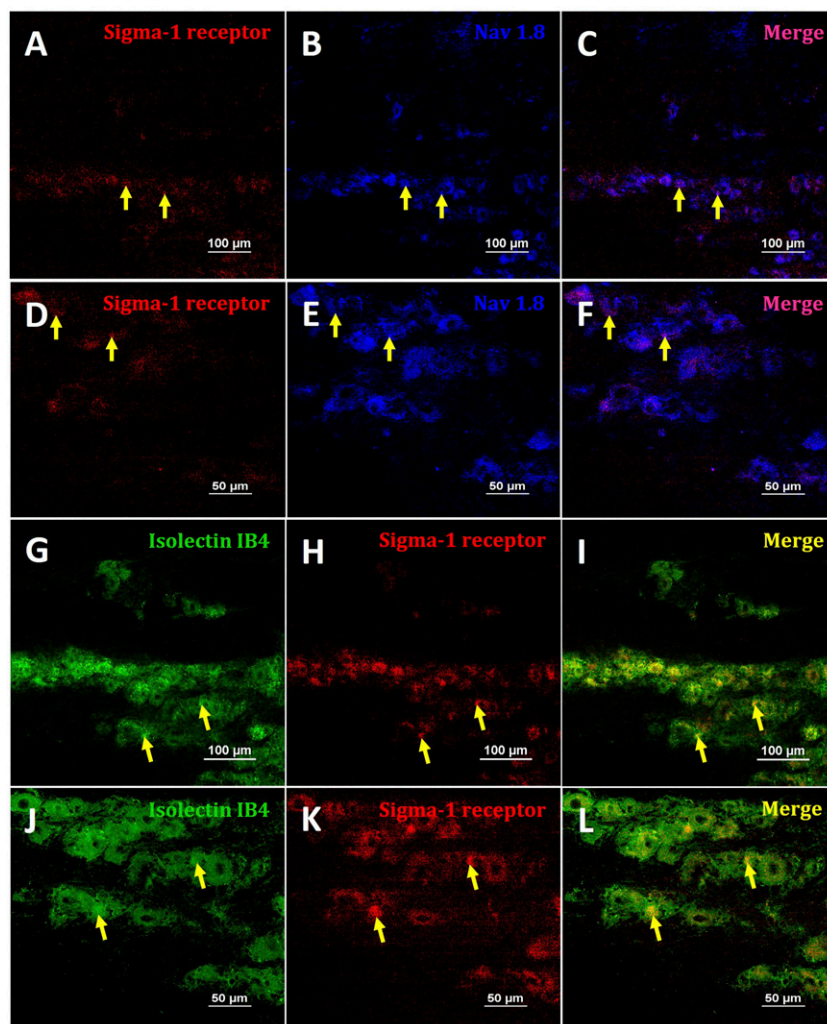


Figure 3. Sigma-1 receptors are expressed in nociceptive and non-peptidergic neurons in the female rat trigeminal ganglia. Immunofluorescent staining of sigma-1 receptor (Sig-1R; red) (A), sodium channel 1.8 (Nav 1.8; nociceptors; blue) (B), and merged image (C) at 20X magnification. Sig-1 R (red) (D), Nav 1.8 (blue) (E), and merged image (F) at 40X magnification. Immunofluorescent staining of Sig-1R (red) (H), isolectin IB4 (non-peptidergic neurons; green) (G), and merged image (I) at 20X magnification. Sig-1 R (red) (K), isolectin IB4 (J), and merge images (L) at 40X magnification. Arrows indicate cells with protein coexpression.

expressed Sig-1R positive (open bars; Figure 12(f)). These data reveal that the nociceptive, peptidergic population of trigeminal sensory neurons highly express Sig-1R.

Progesterone metabolizing enzymes are present in nociceptive neuron populations of the female rat trigeminal ganglia

The progesterone metabolizing enzyme 5α -reductase was detected by western blot in the female rat trigeminal ganglia at each stage of the estrous cycle (Figure 5(a)). The level of 5α -reductase did not significantly vary across the rat estrous cycle [$F(3,12) = 2.171$; $p > 0.05$] (Figure 5(b)). Further, the 3α -HSD enzyme was also detected by western blot in the female rat trigeminal ganglia at each stage of the estrous cycle

(Figure 5(c)). The level of 3α -HSD also did not significantly vary across the rat estrous cycle [$F(3,12) = 0.3112$; $p > 0.05$] (Figure 5(d)). Double immunohistochemical staining of trigeminal ganglia of intact female rats revealed that PGP9.5-positive neurons (Figure 6(a) and (d)) and 5α -reductase-positive cells (Figure 6(b) and (e)) overlapped in immunoreactivity (Figure 6(c) and (f)) with 100% of PGP9.5 immunoreactive cells also being immunoreactive for 5α -reductase (gray bar; Figure 12(a)). Neurons immunoreactive for NF200 (Figure 6(g) and (j)) and cells immunoreactive for 5α -reductase (Figure 6(h) and (k)) also overlapped in labeling (Figure 6(i) and (l)). Again, all NF200-positive cells contained 5α -reductase (gray bar; Figure 12(b)). 5α -reductase immunoreactivity was observed in prostate tissue (positive control) but absent in testes (negative control) (data not shown).

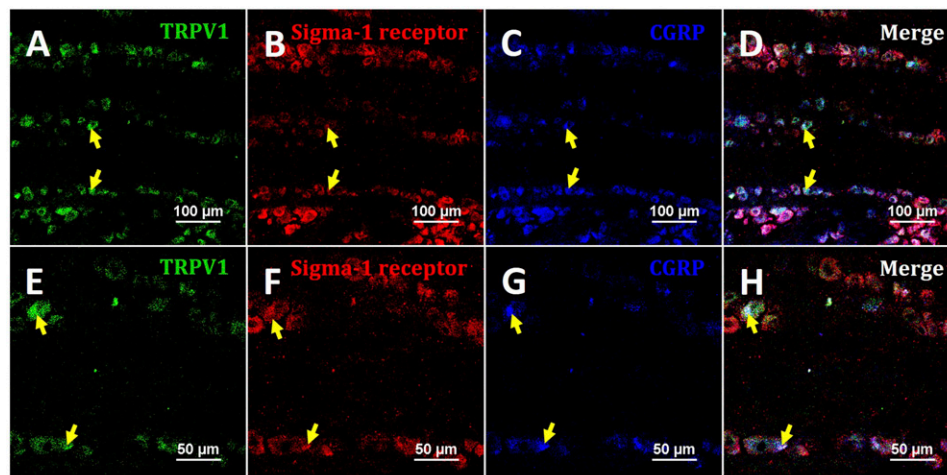


Figure 4. Sigma-1 receptors are expressed in a subpopulation of peptidergic nociceptors within the trigeminal ganglia of female rats. Immunofluorescent staining of the transient receptor potential vanilloid 1 cation channel (TRPV1; nociceptors; green) (A), sigma-1 receptor (Sig-1R; red) (B), calcitonin gene-related peptide (CGRP; peptidergic neurons; blue) (C), and merged image (D) at 20X magnification. TRPV1 (green) (E), Sig-1R (red) (F), CGRP (blue) (G), and merged image (H) at 40X magnification. Arrows indicate cells with protein coexpression.

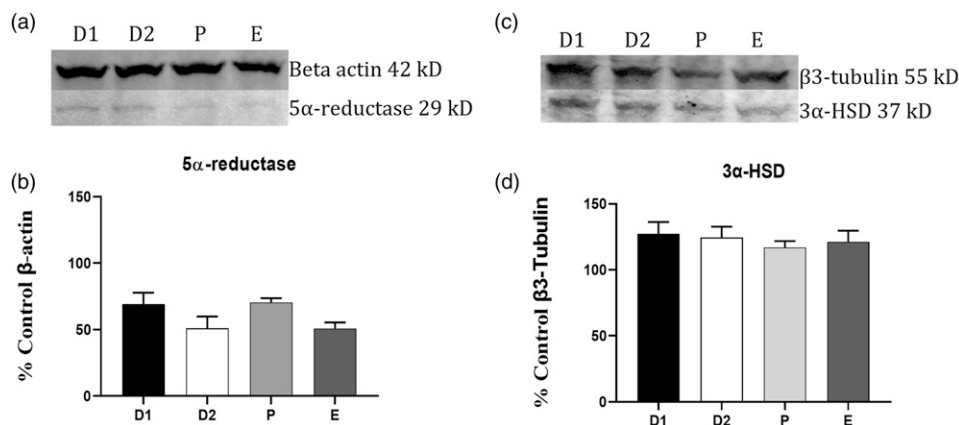


Figure 5. Progesterone metabolizing enzymes, 5 α -reductase and 3 α -hydroxysteroid dehydrogenase, are present in the female rat trigeminal ganglia at similar levels across the estrous cycle. 5 α -reductase enzyme was detected by western blot (A) and quantified (B) across each phase of the estrous cycle. 3 α -hydroxysteroid dehydrogenase (3 α -HSD) was also detected by western blot (C) and quantitated (D) across each phase of estrous cycle [diestrus 1 (D1); diestrus 2 (D2); proestrus (P); estrus (E)]. No significant differences were detected.

These data reveal that trigeminal sensory neurons, including myelinated neurons, contain an enzyme necessary for progesterone metabolism.

Further, expression of the sodium ion channel Nav1.8 identified a subpopulation of trigeminal nociceptive sensory neurons (Figure 7(a) and (d)) and localization of the progesterone metabolizing enzyme 5 α -reductase (Figure 7(b) and (e)) in the Nav1.8-positive population (Figure 7(c) and (f)). About 97% of the Nav1.8 population was 5 α -reductase-positive (gray bar; Figure 12(c)), which provides further evidence that progesterone metabolism occurs in nociceptive trigeminal sensory neurons in the female rat. Non-peptidergic cells labeled with Isolectin IB4 (Figure 7(g) and (j)) and cells

that contained 5 α -reductase enzyme (Figure 7(h) and (k)) were observed to be co-localized in a subpopulation of trigeminal sensory neurons (Figure 7(i) and (l)). Approximately 87% of Isolectin IB4-positive cells were also immunoreactive for 5 α -reductase (gray bar; Figure 12(d)). Immunoreactivity for a subpopulation of nociceptive sensory neurons, the TRPV1 population, was observed in the trigeminal ganglia of female rats (Figure 8(a) and (e)). This population was highly peptidergic, as expected and observed as CGRP immunoreactivity (Figure 8(b) and (f)). Of the TRPV1-positive cells, 94% were also positive for 5 α -reductase (gray bar; Figure 12(e)) and 90% of the CGRP-positive cells contained 5 α -reductase (gray bar; Figure 12(f)). Thus, the 5 α -reductase

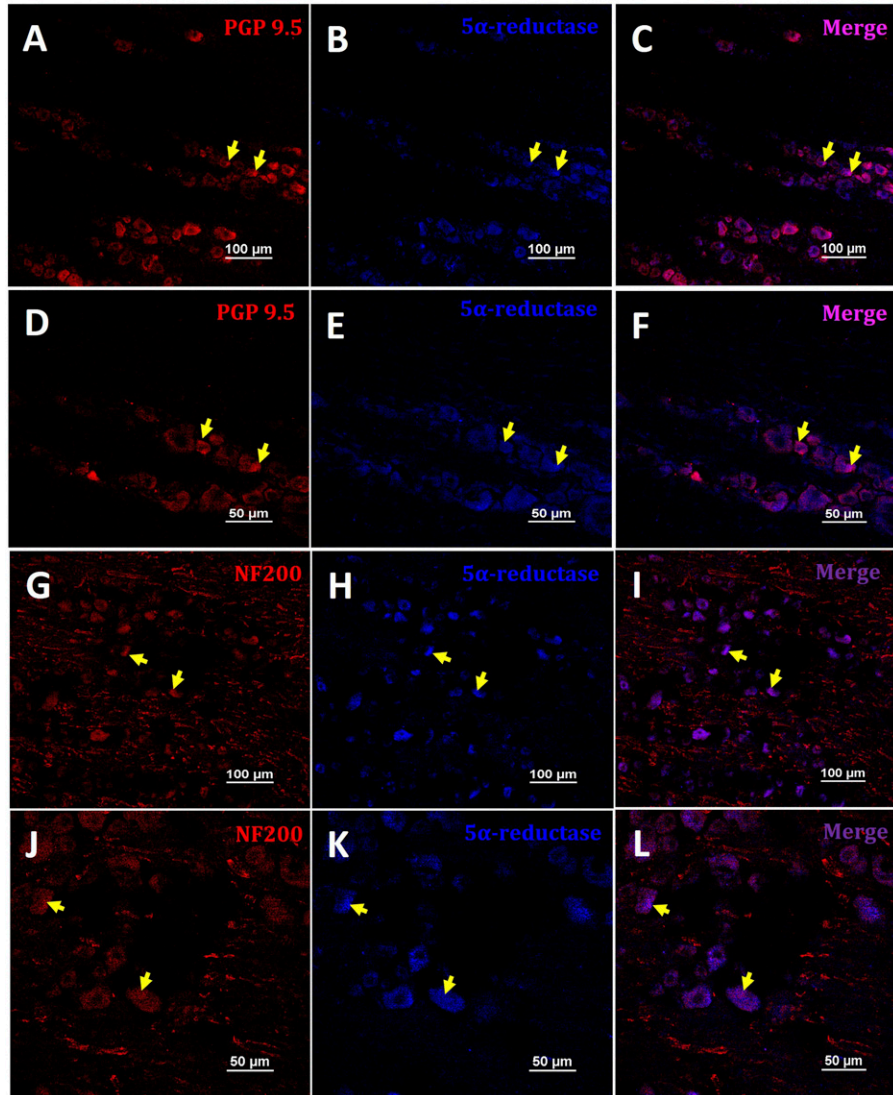


Figure 6. 5α -reductase expression in neurons, nerve fibers, and myelinated neurons within the trigeminal ganglia of female rats. Immunofluorescent staining of protein gene product 9.5 (PGP9.5; nerve fibers and neurons; red) (A), 5α -reductase (blue) (B), and merged image (C) at 20X. PGP9.5 (red) (D), 5α -reductase (blue) (E), and merge images (F) at 40X magnification. Immunofluorescent staining of neurofilament heavy (NF200; myelinated neurons; red) (G), 5α -reductase (blue) (H), and merged image (I) at 20X magnification. NF200 (red) (J), 5α -reductase (blue) (K), and merged image (L) at 40X magnification. Arrows indicate cells with overlapping protein and enzyme immunoreactivity.

enzyme (Figure 8(c) and (g)) was expressed in a subpopulation of peptidergic, TRPV1-positive female rat trigeminal sensory neurons (Figure 8(d) and (h)).

Similar findings were observed for another progesterone metabolizing enzyme 3α -hydroxysteroid dehydrogenase (3α -HSD). Double immunohistochemical staining of the trigeminal ganglia of intact female rats revealed that PGP9.5-positive neurons (Figure 9(a) and (d)) and 3α -HSD-positive cells (Figure 9(b) and (e)) overlapped in immunoreactivity (Figure 9(c) and (f)), such that 96% of the PGP9.5 population were also immunoreactive for

3α -HSD (closed bars; Figure 12(a)). Neurons immunoreactive for NF200 (Figure 9(g) and (j)) and cells immunoreactive for 3α -HSD (Figure 9(h) and (k)) also overlapped in labeling (Figure 9(i) and (l)). All of NF200-positive neurons were also immunoreactive for 3α -HSD (closed bars; Figure 12(b)). 3α -HSD immunoreactivity was observed in prostate tissue (positive control) but absent in testes (negative control) (data not shown). Together these data reveal that trigeminal sensory neurons, including myelinated neurons, contain two enzymes necessary for progesterone metabolism.

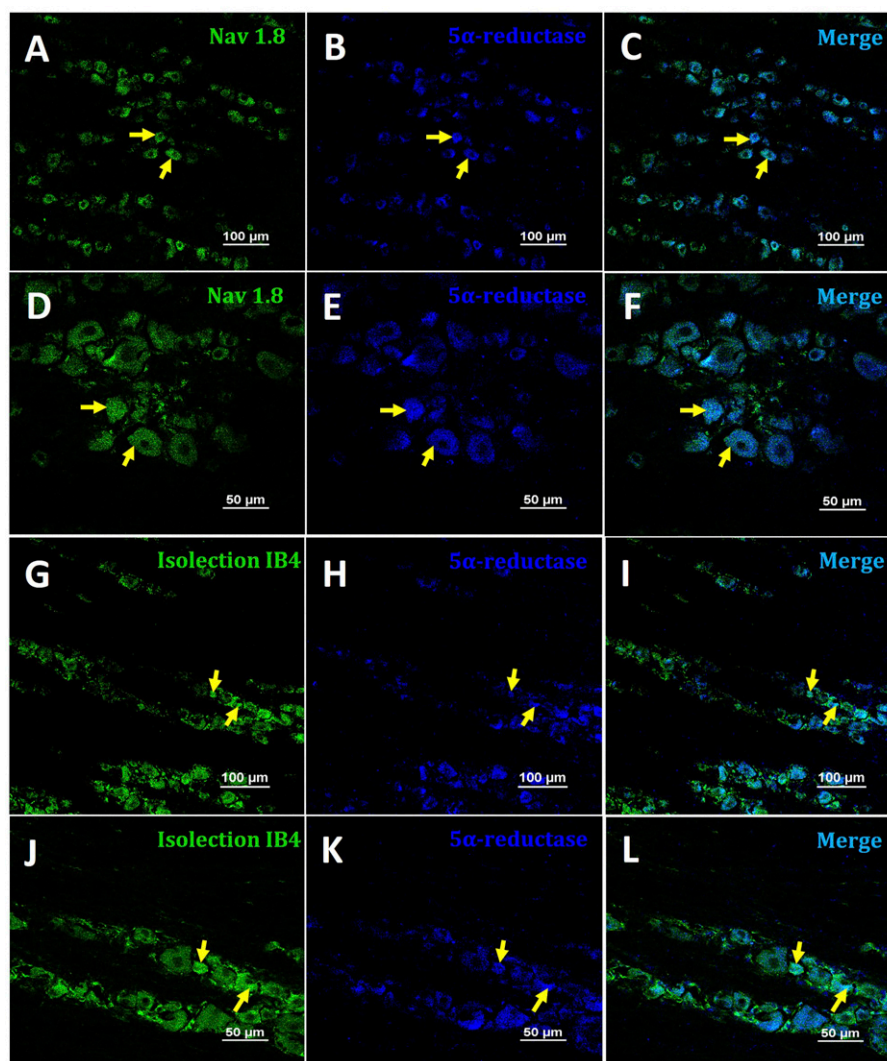


Figure 7. 5α -reductase expression in non-peptidergic nociceptive sensory neurons within the trigeminal ganglia of female rats. Immunofluorescent staining of sodium channel 1.8 (Nav 1.8; nociceptor; green) (A), 5α -reductase (blue) (B), and merged image (C) at 20X magnification. Nav 1.8 (green) (D), 5α -reductase (blue) (E), and merged images (F) at 40X magnification. Immunofluorescent staining of isolectin IB4 (non-peptidergic neurons; green) (G), 5α -reductase (blue) (H), and merged image (I) at 20X magnification. Isolectin IB4 (green) (J), 5α -reductase (blue) (K), and merged image (L) at 40X magnification. Arrows indicate cells with overlapping protein and enzyme immunoreactivity.

Further, expression of the sodium ion channel Nav1.8 identified a subpopulation of trigeminal nociceptive sensory neurons (Figure 10(b) and (e)) and localization of progesterone metabolizing enzyme 3α -HSD (Figure 10(a) and (d)) in the Nav1.8-positive population (Figure 10(c) and (f)) provides further evidence that progesterone metabolism occurs in female rat trigeminal nociceptors. Approximately 99% of the Nav1.8 population was also immunoreactive for 3α -HSD (closed bars; Figure 12(c)). Non-peptidergic cells labeled with Isolectin IB4 (Figure 10 (g) and (j)) and cells that contained 3α -HSD enzyme (Figure 10(h) and (k)) were observed to be highly co-localized (Figure 10(i) and (l)). Of this non-peptidergic trigeminal neuron population, approximately 94% were 3α -HSD-positive (closed bar; Figure 12(d)).

Immunoreactivity for TRPV1 was observed in the trigeminal ganglia of female rats (Figure 11(a) and (e)). This population was again highly peptidergic, observed as CGRP immunoreactivity (Figure 11(c) and (g)). The 3α -HSD enzyme (Figure 11(b) and (f)) was expressed in a subpopulation of peptidergic, TRPV1-positive trigeminal sensory neurons (Figure 11(d) and (h)). All TRPV1-positive cells were also positive for 3α -HSD (closed bar; Figure 12(e)) and 98% of the CGRP-positive cells contained 3α -HSD (closed bar; Figure 12(f)).

Discussion

Previously, we reported the rapid attenuation of high estradiol-evoked mechanical allodynia by two different doses

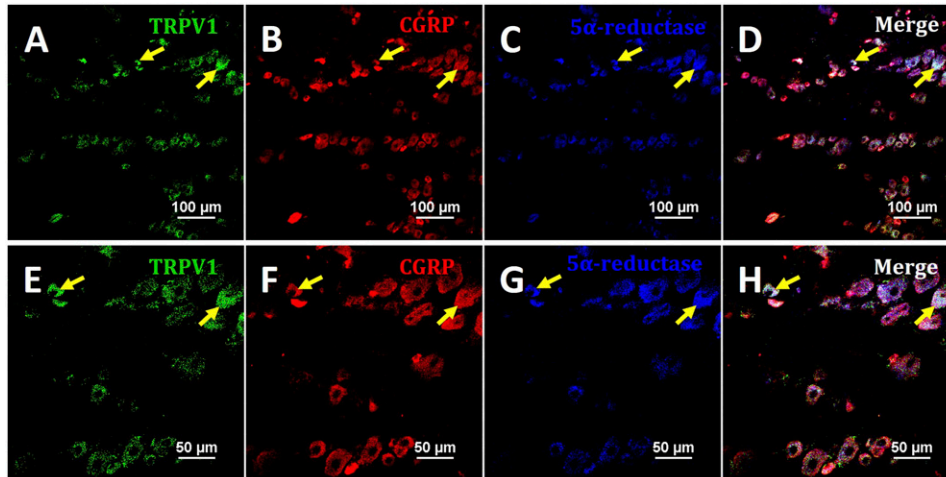


Figure 8. 5α -reductase expression in a subpopulation of peptidergic nociceptive sensory neurons within the trigeminal ganglia of female rats. Immunofluorescent staining of transient receptor potential vanilloid I (TRPV1; subtype of nociceptor; green) (A), calcitonin gene-related peptide (CGRP; peptidergic neurons; red) (B), 5α -reductase (blue) (C), and merged image (D) at 20X magnification. TRPV1 (green) (E), CGRP (red) (F), 5α -reductase (blue) (G), and merged image (H) at 40X magnification. Arrows indicate cells with overlapping protein and enzyme immunoreactivity.

of progesterone, as well as the acute, rapid attenuation by the progesterone metabolite, allopregnanolone, in female rats with persistent temporomandibular joint inflammation.³⁷ The orofacial region is innervated by the trigeminal nerve, which has nociceptors that are sensitive to noxious chemical, mechanical, and thermal stimuli. Nociceptors innervating the cranio-orofacial region have cell bodies located in the trigeminal ganglia and are excited by noxious chemical, mechanical, and thermal stimuli. Excitation of the nociceptors is relayed to the trigeminal nucleus subcaudalis in the medullary spinal cord, which transmits the signal on to the thalamus and somatosensory cortex.⁵⁸ Progesterone's protective mechanisms can potentially occur within any of the peripheral and central nervous system anatomical locations mentioned above. Within the trigeminal system, estrogen upregulates inflammatory mediators,^{25,59,60} ion channels,^{26,29,61,62} and increases excitability of sensory neurons.^{27,63,64} Although most studies focus on estrogen's effects on nociception, several have investigated progesterone's effects on the trigeminal system. Data from these studies indicates an antinociceptive role for progesterone within the trigeminal system.^{22,37,65}

Of the ovarian hormones, estrogen and progesterone, there is extensive evidence supporting a protective role for progesterone in nervous system diseases, disorders, and injuries. Although progesterone is known to be antinociceptive, the anatomical substrate for progesterone's attenuation of inflammatory orofacial pain has not been clearly identified. Two major mechanisms known to have a role in progesterone's antinociceptive effects are the Sig-1R and allopregnanolone. Progesterone is an antagonist at Sig-1R, but through metabolism to allopregnanolone, progesterone can

indirectly potentiate GABA_A receptors. Conversion of progesterone to allopregnanolone requires two enzymes, 5α -reductase and 3α -HSD. Progesterone is first metabolized to 5α -dihydroprogesterone by 5α -reductase. 5α -dihydroprogesterone is then converted to allopregnanolone by the aldo-keto reductase, 3α -hydroxysteroid dehydrogenase. None of the neuroanatomical targets mentioned here have been reported in the female trigeminal ganglia. In the current study, we hypothesized that Sig-1R and the progesterone metabolizing enzymes are present in nociceptive sensory neurons of the female rat trigeminal ganglia as a potential anatomical substrate for the antinociceptive actions of progesterone. Here, we are the first to report that the Sig-1R, 5α -reductase, and 3α -HSD are found in nociceptive sensory neuron populations of the trigeminal ganglia of intact, naturally cycling female rats and do not vary across the estrous cycle.

Sig-1R is a non-opioid endoplasmic reticulum chaperone protein located within the mitochondrial-associated endoplasmic reticulum membrane⁶⁶ and highly expressed in several pain-related areas, including the dorsal root ganglia (DRG), periaqueductal gray, thalamus, and basolateral amygdala.⁶⁷⁻⁶⁹ Our data contributes the finding that Sig-1R are highly expressed in the trigeminal ganglia of female rats and expression levels do not appear to be altered by fluctuating gonadal hormones. Sig-1Rs are found in both the TRPV1 and Nav1.8 subpopulations of sensory neurons that can be identified as nociceptors.⁷⁰ Further, we found that Sig-1R are found in myelinated (NF200+) neurons. Progesterone is known to have promyelinating effects within the nervous system,⁷¹ which may attenuate pain associated with demyelinating diseases, such as Charcot-Marie tooth disease or

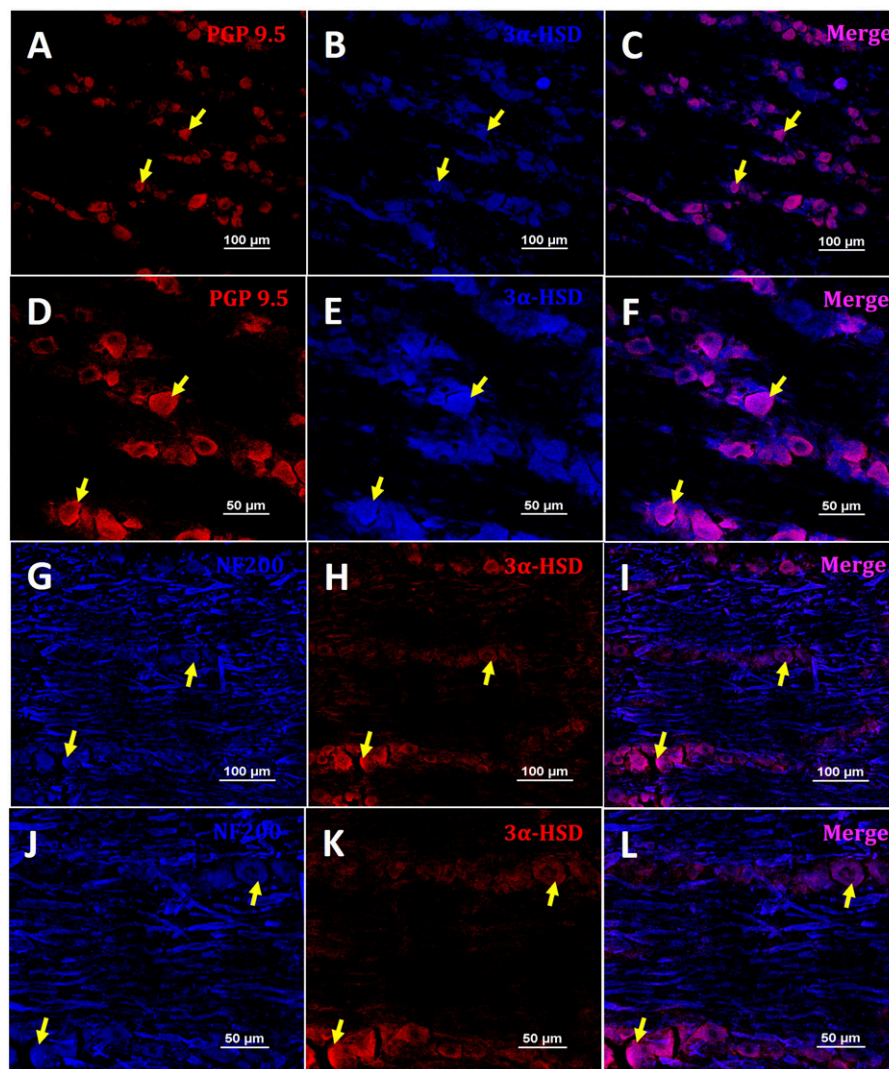


Figure 9. 3α -hydroxysteroid dehydrogenase is expressed in neurons, nerve fibers, and myelinated neurons within the trigeminal ganglia of female rats. Immunofluorescent staining of protein gene product 9.5 (PGP9.5; neurons and nerve fibers; red) (A), 3α -hydroxysteroid dehydrogenase (3α -HSD; blue) (B), and merged image (C) at 20X magnification. PGP9.5 (red) (D), 3α -HSD (blue) (E), and merge images (F) at 40X magnification. Immunofluorescent staining of neurofilament heavy (NF200; myelinated neurons; blue) (G), 3α -hydroxysteroid dehydrogenase (3α -HSD; red) (H), and merged image (I) at 20X magnification. NF200 (blue) (J), 3α -HSD (red) (K), and merged images (L) at 40X magnification. Arrows indicate cells with overlapping protein and enzyme immunoreactivity.

multiple sclerosis where patients experience “burning” and “stabbing” pain.^{72,73}

Nociceptors can also be peptidergic (CGRP-positive) and non-peptidergic (IB4-positive). Since CGRP is expressed in some non-nociceptive cells, we particularly highlight that Sig-1Rs are found in the CGRPergic TRPV1 population. Progesterone consistently downregulates inflammatory mediators,⁷⁴⁻⁷⁶ including the proinflammatory and pronociceptive mediator CGRP.⁷⁷ In support, progesterone deficiency augments CGRP activity.⁷⁸ Thus, progesterone could potentially reduce CGRP activity within the trigeminal ganglia to contribute to the reduction in orofacial pain

observed in our previous study.³⁷ CGRP is released upon TRPV1 activation⁷⁹ and CGRPergic TRPV1 neurons are highly expressed within the trigeminal ganglia.⁸⁰ Our findings contribute evidence that the CGRPergic TRPV1 population in female rats contains targets for progesterone. In support, previous studies report Sig-1R agonists facilitate capsaicin-induced mechanical allodynia,⁴⁹ which is reversed by Sig-1R antagonists.⁸¹⁻⁸³ Additionally, our data support previous research reporting that Sig-1R colocalizes with TRPV1 in the DRGs of male and female mice and progesterone reduces TRPV1 expression.⁶⁹ Non-peptidergic nociceptors have also been shown to express Sig-1R in the DRGs

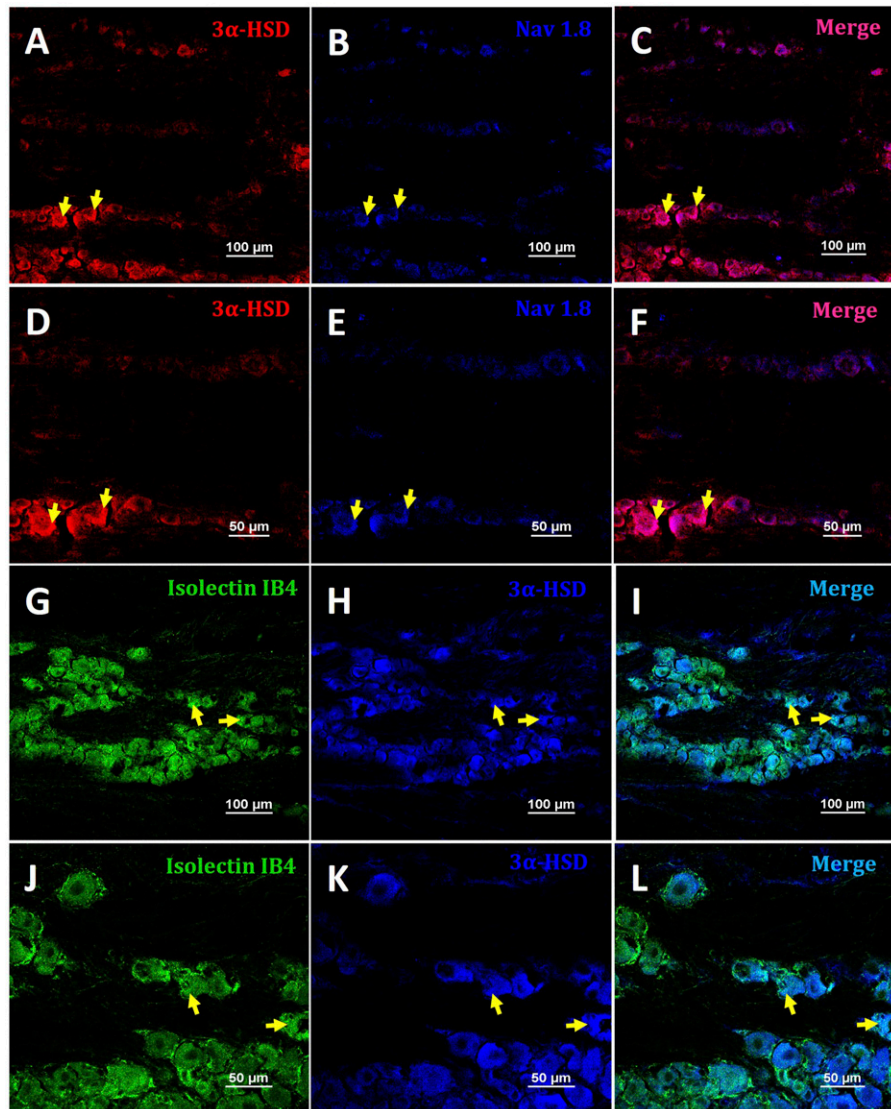


Figure 10. 3α -hydroxysteroid dehydrogenase expression in non-peptidergic nociceptive sensory neurons within the trigeminal ganglia of female rats. Immunofluorescent staining of sodium channel 1.8 (Nav 1.8; nociceptor; blue) (B), 3α -hydroxysteroid dehydrogenase (3α -HSD; red) (A), and merged image (C) at 20X magnification. Nav 1.8 (blue) (E), 3α -HSD (red) (D), and merged images (F) at 40X magnification. Immunofluorescent staining of isolectin IB4 (non-peptidergic neurons; green) (G), 3α -HSD (blue) (H), and merged image (I) at 20X magnification. Isolectin IB4 (J), 3α -HSD (blue) (K), and merged image (L) at 40X magnification. Arrows indicate cells with overlapping protein and enzyme immunoreactivity.

of male rats⁶⁸ and here we add that non-peptidergic nociceptors in the trigeminal ganglia of female rats also express Sig-1R.

Our previous study also reported that conversion to allopregnanolone by the enzymes 5α -reductase and 3α -HSD contributes to the antinociceptive properties of progesterone on orofacial pain,³⁷ as allopregnanolone is a positive allosteric modulator of the GABA_A receptor. In support, progesterone metabolites and allopregnanolone can attenuate nociceptive behaviors.⁸⁴ 5α -reductase and 3α -HSD are expressed within the central nervous system,^{85,86} the peripheral

nervous system within the sciatic nerve,⁸⁷ and in the DRG.⁴⁰ Here, we contribute to the known locations of 5α -reductase and 3α -HSD to include the nociceptive sensory neuron populations of the female rat trigeminal ganglia.

5α -reductase and 3α -HSD enzymes were found within peptidergic, non-peptidergic, and myelinated trigeminal ganglia neurons. Both enzymes were found in the Nav1.8 and TRPV1 subpopulations of nociceptive trigeminal sensory neurons. Expression in myelinated neurons was not surprising since both enzymes are found in male and female rat oligodendrocytes^{85,88} and both alter myelin protein

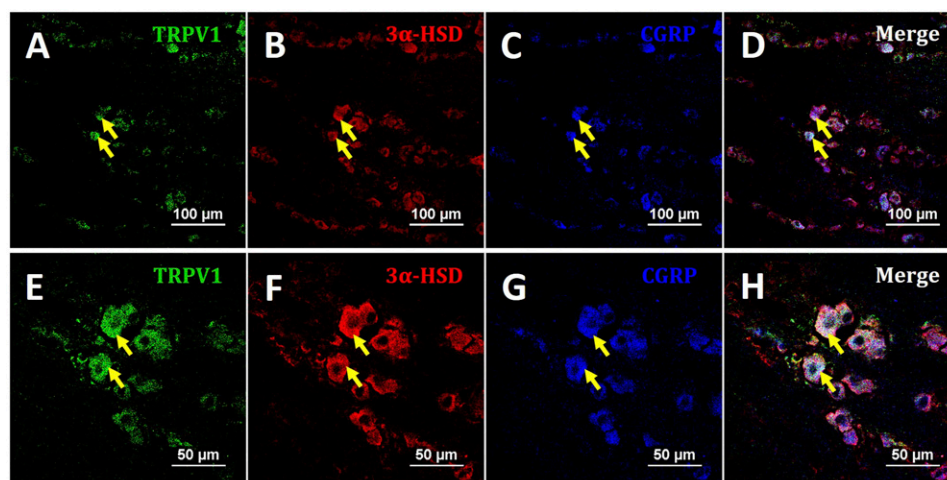


Figure 11. 3α -hydroxysteroid dehydrogenase expression in a subpopulation of peptidergic nociceptive sensory neurons within the trigeminal ganglia of female rats. Immunofluorescent staining of transient receptor potential vanilloid I (TRPV1; subtype of nociceptor; green) (A), 3α -hydroxysteroid dehydrogenase (3α -HSD; red) (B), calcitonin gene-related peptide (CGRP; peptidergic neurons; blue) (C), and merged image (D) at 20X magnification. TRPV1 (green) (E), 3α -HSD (red) (F), CGRP (blue) (G), and merged image (H) at 40X magnification. Arrows indicate cells with overlapping protein and enzyme immunoreactivity.

expression.⁸⁹ Since allopregnanolone has antinociceptive effects,⁸⁴ expression of 5α -reductase and 3α -HSD in nociceptive sensory neurons provides evidence that pain reduction can occur at the trigeminal ganglia.

Although the present study did not compare the levels or localization of Sig-1R or the enzymes between males and females, future studies are warranted given known sex differences in pain and sex differences in the prevalence of a variety of pain disorders. This comparison would help to determine any sex-specific mechanisms that may contribute to sex differences in pain. Further the present study focused on CGRPergic neurons, while another neuropeptide, substance P, may be involved in the antinociceptive effects of progesterone. Substance P has been shown to inhibit progesterone metabolism within the spinal cord, thus resulting in a decrease in circulating allopregnanolone.⁹⁰ If substance P can decrease progesterone metabolism in the trigeminal ganglia, then progesterone treatment may counter the drop in antinociceptive levels of allopregnanolone. Nevertheless, allopregnanolone is a positive allosteric modulator of the GABA_A receptor, which is expressed in the trigeminal ganglia,^{59–77,79–91} therefore, allopregnanolone may enhance GABA-mediated antinociception. This is an interesting avenue for exploration as GABA_A receptors are a potential progesterone target in female prevalent chronic pain conditions and expressed in male and female rodent and human sensory ganglia^{91–95} with sex differences in nociceptive function reported^{96,97} potentially due to alteration of subunit expression by chronic pain^{92,93} Future studies to investigate manipulation of these enzymes and observe their effects on orofacial pain behaviors in both male and

female subjects are warranted, and our findings provide further evidence that GABA receptors in sensory ganglia may provide a novel therapeutic target for pain.

The trigeminal ganglia are not the only potential anatomical substrate for progesterone's antinociceptive actions on orofacial pain. The trigeminal nucleus caudalis in the medullary spinal cord is also a likely target. CGRP⁹⁸ and TRPV1⁹⁹ are both expressed in the trigeminal nucleus caudalis and progesterone reduces both CGRP and TRPV1 levels at this brainstem site¹⁰⁰ to contribute to a reduction in pain. Chronic activation of the Sig-1R results in evoked nociception by activating the trigeminal nucleus caudalis,⁴⁷ thus progesterone-induced reduction in CGRP or TRPV1 at the brainstem may reduce pain. Additionally, GABA_A receptors are expressed in the trigeminal subnucleus caudalis¹⁰¹ where allopregnanolone could enhance GABA-mediated antinociception. Future studies should also focus on mechanisms for progesterone's activity within the trigeminal nucleus caudalis.

Overall, we provide evidence that Sig-1Rs are available to progesterone in the trigeminal ganglia of female rats in similar concentrations across the estrous cycle and that progesterone could be metabolized locally in trigeminal sensory neurons as the required enzymes are present. The trigeminal ganglia are thus an anatomical substrate for the antinociceptive actions of progesterone and its metabolite allopregnanolone on orofacial or craniofacial pain. Future studies directly targeting progesterone's antinociceptive mechanisms within the trigeminal ganglia and observing trigeminal pain behaviors are warranted by our neuroanatomical findings. Improving knowledge on progesterone's modes of action, especially for pain disorders that are more prevalent in women, may lead to sex-specific therapeutics for women in pain.

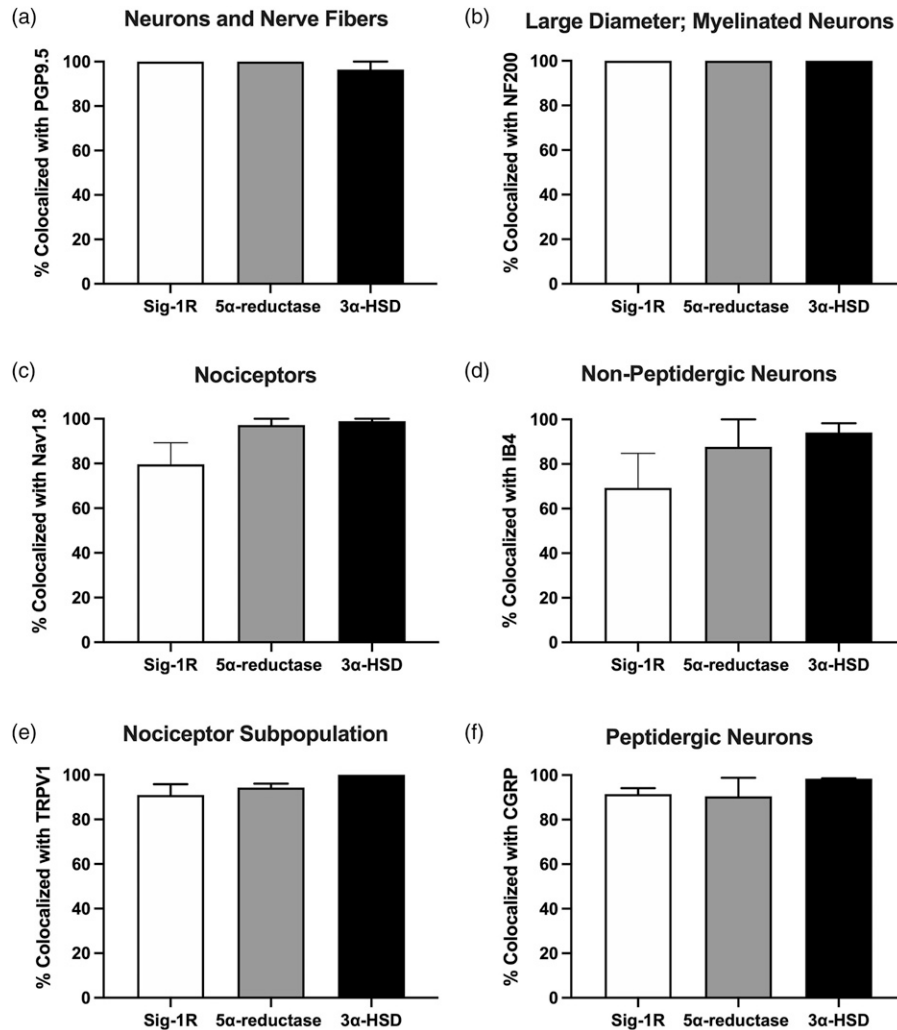


Figure 12. Sigma-1 receptors and progesterone metabolizing enzymes, 5 α -reductase and 3 α -hydroxysteroid dehydrogenase, are highly expressed across various neuron populations in female rat trigeminal ganglia neurons. Quantification of the percentage of Sigma-1 receptors (Sig-1R), 5 α -reductase, or 3 α -hydroxysteroid dehydrogenase (3 α -HSD) that are co-localized within the following neuron populations: protein gene product 9.5 (PGP9.5; neurons and nerve fibers; A), neurofilament heavy (NF200; myelinated neurons; B), sodium channel 1.8 (Nav1.8; nociceptors; C), isolectin IB4 (IB4, non-peptidergic neurons; D), transient receptor potential vanilloid 1 (TRPV1; nociceptor subpopulation; E), or calcitonin gene-related peptide (CGRP; peptidergic neurons; F).

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Author Contributions

RSH contributed to experimental design, conducting experiments, data analysis and interpretation, and preparation of the manuscript. NGR contributed to conducting experiments, data analysis and interpretation, and approval of the manuscript. DJC contributed to conducting experiments, data analysis, and approval of the manuscript. LL contributed to data analysis and approval of manuscript. DA contributed to experimental design, data analysis and interpretation, and preparation of the manuscript.

Declaration of conflicting interests

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