ESTROGEN SIGNALING IN TRIGEMINAL NOCICEPTION

by

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List of Abbreviations

CFA ……..……Complete Freund's Adjuvant

TMD……...Temporomandibular Disorder

E2……..... 17β-Estradiol

GPR30…….. G-Protein coupled Receptor 30

ERα…….....Estrogen Receptor α

ERβ……..... Estrogen Receptor β

TMJ……...……...Temporomandibular Joint

NFH……...Neurofilament H

OVX……ovariectomized

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Abstract

Migraine is much more common in women than in men, and painful episodes are linked to the hormonal fluctuations of the menstrual cycle. The MAP kinase extracellular-signal regulated kinase (ERK) is activated in experimental models of chronic pain, and is also activated by estrogen in sensory neurons. We used an established model of inflammatory trigeminal pain, injection of Complete Freund's adjuvant (CFA) into the masseter muscle, to determine whether ERK activation may play a role in hormone-related trigeminal pain disorders. We measured withdrawal responses to stimulation of the masseter (V3, primary allodynia) and whisker pad (V2, secondary allodynia) using graded monofilaments. Estrogen treatment in the presence of inflammation increased withdrawal response to stimulation of either masseter or whisker pad compared to inflammation alone, indicating an additive effect of inflammation and estrogen on both primary and secondary allodynia. We examined ERK activation in trigeminal ganglia from each treatment group using Western blot and immunohistochemistry. Both masseter inflammation and estrogen treatment increased ERK activation, and combined treatment had an additive effect. Both masseter inflammation and estrogen increased the percentage of pERK immunoreactive neurons in divisions 1 and 2 (V1/2), and combined treatment increased pERK immunoreactivity in V1/2 compared to inflammation alone. We stereotactically administered ERK antagonist U0126, or inactive control U0124, to the trigeminal ganglion of CFA+E2-treated rats. U0126 decreased withdrawal responses to mechanical stimulation of the whisker pad compared to U0124-treated rats. Because the secondary allodynia in V2 after inflammation in V3 was reduced by

antagonizing ERK activation in the periphery, these data suggest a peripheral component to secondary trigeminal allodynia mediated through ERK activation.

Cellular responses to estrogen can occur through both 'genomic' and 'non-genomic' pathways. The novel estrogen receptor GPR30 (G-protein coupled receptor 30) activates mediators of signal transduction, including ERK. A goal of this study was to determine which estrogen receptor is required for behavioral sensitization. In order to determine whether GPR30 is present in neurons of the trigeminal ganglion, we performed Western blots for GPR30 on trigeminal ganglion from ovariectomized female Sprague-Dawley rats. Our results show the presence of GPR30 protein in the trigeminal ganglion. Immunohistochemistry for GPR30 in trigeminal ganglion sections showed that GPR30 immunoreactivity was localized to neuronal cell bodies. To determine the subpopulation of neurons that express GPR30, we measured the diameters of GPR30 positive and negative neurons. Neurons expressing GPR30 were significantly smaller in diameter, suggesting that GPR30 is present in nociceptors. In order to investigate GPR30 expression within sub-populations, we co-localized GPR30 with peripherin and NFH using double label immunohistochemistry. GPR30 showed a high degree of overlap with peripherin and partial overlap with NFH, indicating that GPR30 is preferentially expressed in neurons with unmyelinated axons.

ERK activation by estrogen in the trigeminal ganglion may occur through either the classical estrogen receptor ERα or through GPR30. In order to determine which estrogen receptor mediates ERK activation in trigeminal ganglion neurons, we examined ERK activation in primary cultures of trigeminal ganglion neurons treated with selective agonists for ERα (PPT), ERβ (DPN), and GPR30 (G-1). ERK was

activated by selective agonists for ERα and GPR30, suggesting that activation occurs through either receptor. In order to determine which estrogen receptor mediates increased trigeminal sensitivity, we used a previously employed behavioral model of inflammatory trigeminal allodynia. Ovariectomized female Sprague-Dawley rats were injected in the masseter with CFA and subcutaneously administered G-1, PPT, or vehicle. Withdrawal response to stimulation of the whisker pad (V2) was measured using monofilaments. Either G-1 or PPT treatment increased secondary allodynia, indicating that both receptors function in trigeminal sensitization in vivo. Treatment with estrogen increased expression of ERα but not GPR30, while masseter inflammation increased GPR30 but not ERα. Differential modulation of these ERK-coupled receptors by estrogen and inflammation may play a role in increased trigeminal pain during periods of falling estrogen.

I. Background and Significance

Introduction

Disorders of trigeminal pain are among the most common illnesses and present a significant burden to the individual and to society. Migraine headache is the most common neurological disorder in the world, affecting 12%-15% of the population. A related disorder, temporomandibular disorder (TMD), which is characterized by episodes of pain in the temporomandibular joint and muscles of mastication, affects 7-15% of the adult population (Goulet et al., 1995). Migraine is estimated to be the most costly neurological disorder, with an estimated annual cost of $16.6 billion in the US and €27 billion in Europe (Andlin-Sobocki et al., 2005). Severe migraine is classified as one of the most disabling illnesses by the World Health Organization (Menken et al., 2000) and trigeminal pain disorders are estimated to account for 9% of lost productivity (Stewart et al., 2003). Because the onset of migraine occurs before the age of 30 in 80% of cases and occurs chronically over many years, it carries a high level of morbidity. Migraine and related trigeminal disorders are clearly significant problems that merit basic research.

Migraine and temporomandibular disorder are both more prevalent in women, a characteristic that may be important in understanding their etiology and pathology. Migraine is three times more prevalent in women than in men; (Locker and Slade, 1988; Dworkin et al., 1990; Lipton et al., 1993; Stewart et al., 1994; Yunus, 2002) and has a lifetime prevalence of 25% in women (Rasmussen, 1995). Temporomandibular joint disorders are at least twice as common in women than in men, and 80% of individuals seeking treatment for TMD are women (Dworkin et al., 1990). Women experience facial pain primarily in the years between menarche and

menopause. Both migraine and temporomandibular disorder commonly begin around the time of menarche, increase during the reproductive years, and regress after menopause (LeResche et al., 2005; Lipton and Bigal, 2005), suggesting that estrogen fluctuations play a role in these disorders. Despite substantial clinical data, the mechanisms of estrogen's role in trigeminal pain remain unclear and warrant further investigation.

Clinical aspects of migraine

The most common and well studied trigeminal pain disorder is migraine headache. The migraine episode is a complex constellation of symptoms that characteristically involves a severe, unilateral headache with a throbbing or pulsating character. The headache is associated with nausea and sensitivity to light and sound, referred to as photophobia, and phonobobia, respectively. Some patients experience auras, disturbances in the visual field, or other sensory phenomena, that directly precede the onset of headache. For this reason, migraine is divided into two major categories—migraine without aura and migraine with aura, formerly known as common migraine and classic migraine, respectively. Migraine without aura (common migraine) is more common, with an incidence five times that of migraine with aura (Rasmussen, 1995). Although migraine with aura and migraine without aura are regarded as distinct disorders (Russell et al., 1996), some patients experience both migraine with aura and migraine without aura comorbidly.

Migraine without aura

Migraine without aura progresses as three phases: premonitory, headache and postdrome phases. Premonitory symptoms, which occur between two days and two hours prior to the onset of headache, are predictive of the onset of migraine without aura (Giffin et al., 2003). A headache diary study found that the most commonly reported premonitory symptoms are fatigue, difficulty concentrating and stiff neck. Patients report excitatory states, such as feelings of euphoria or irritability, hyperactivity, cravings, thirst, obssessional behavior, hyperactive bowel

and bladder, or inhibitory states, which include fatigue, mental slowness, slurred speech, anorexia, intolerance to cold, and constipation (Zagami and Bahra, 2005).

During the headache phase, patients experience pain localized to the frontotemporal and ocular regions (Sjaastad et al., 1993). The majority of headaches are unilateral, although bilateral or generalized headaches occur in up to 40% of cases (Campbell, 1990). Pain is described as having a throbbing or pulsating character in up to 82% of reported cases (Russell and Olesen, 1996b) and is often exacerbated by even minor physical activity (Blau and Dexter, 1981). This is a specific characteristic of migraine that differentiates it from other types of headache (Iversen et al., 1990) and is a major reason for its highly disabling nature.

Non-headache symptoms accompany migraine without aura and include nausea, photophobia, and phonophobia in more than 95% of cases (Rasmussen and Olesen, 1992). Non-headache symptoms in the migraineur may be caused by increased sensitivity to non-noxious stimuli (Goadsby, 2001).

Migraine lasts between 4 and 72 hours in 70% of cases (Olesen, 2004). Following resolution of the headache, most patients experience non-specific symptoms for approximately 24 hours in what is known as the post-drome, or 'migraine hangover.' These include changes in mood, fatigue, muscular weakness and anorexia (Blau, 1982).

The diagnostic criteria for migraine *without* aura, as defined by the International Headache Society (IHS), are as follows:

A. At least 5 attacks fulfilling criteria B–D
B. Headache attacks lasting between 4 and 72 hours (untreated or unsuccessfully treated)

C. Headache has at least two of the following characteristics:

1. unilateral location
2. pulsating quality
3. moderate or severe pain intensity
4. aggravation by or causing avoidance of routine physical activity

D. During headache at least one of the following:

1. nausea and/or vomiting
2. photophobia or phonophobia

E. Not attributed to another disorder

Migraine with aura

Migraine with aura is clinically similar to migraine without aura, with the added feature that the headache is immediately preceded by aura. Aura refers to a constellation of neurological symptoms that may include visual, sensory, language, or motor changes. Aura symptoms develop slowly over several minutes and persist for less than one hour (Russell et al., 1994).

Visual aura is reported in 99% of cases and may present in a variety of forms: photopsia—flashes of white, silver, or multicolored light; scintillating scotoma—a blind spot with a shimmering border; or teichopsia—patterns of zigzag lines. Positive visual symptoms may be followed by visual scotoma, or loss of part of the visual field. Visual symptoms are usually confined to one hemifield, although they may occur bilaterally (Russell and Olesen, 1996a)

Non-visual sensory phenomena, most commonly parasthesias, accompany aura in 30-40% of patients (Manzoni et al., 1985). Like visual auras, those skin sensations, such as burning, prickling, itching, or tingling, with no apparent physical

cause are followed by negative symptoms, such as numbness. Non-visual auras tend to be unilateral and involve the perioral region, tongue, and the upper extremity (Manzoni et al., 1985). They are migratory, like visual aura, and progress in a wave along the skin. Language aura presents as a speech disturbance and is reported less frequently than sensory aura (Olesen et al., 1990). It is not clear whether the speech deficit represents cortical aphasia or dysarthria stemming from paralysis of the tongue. Cortical aphasia is the partial or total loss of the ability to communicate orally while dysarthria is difficulty in articulating words due to emotional stress or to paralysis, incoordination, or spasticity of the muscles used in speaking.

The diagnosis of migraine *with* aura as defined by the IHS are as follows:

A. At least 2 attacks fulfilling criteria B–D

B. Aura consisting of at least one of the following, but no motor weakness:

1. fully reversible visual symptoms including positive features (*eg*, flickering lights, spots or lines) and/or negative features (*ie*, loss of vision)
2. fully reversible sensory symptoms including positive features (*ie*, pins and needles) and/or negative features (*ie*, numbness)
3. fully reversible dysphasic speech disturbance

C. At least two of the following:

1. homonymous visual symptoms and/or unilateral sensory symptoms
2. at least one aura symptom develops gradually over ≥5 minutes and/or different aura symptoms occur in succession over ≥5 minutes
3. each symptom lasts ≥5 and <60 minutes

D. Headache fulfilling criteria B–D for *Migraine without aura* begins during the aura or follows aura within 60 minutes

E. Not attributed to another disorder

Aura is thought to be caused by cortical spreading depression, a phenomenon in which cerebral cortical blood flow is transiently decreased to non-ischemic (oligemic) levels. Oligemia travels in a wave across the cortex, usually in an anterior to posterior direction, preceded by a wave of hyperemia. There is controversy in the migraine field about the role of cortical spreading depression in migraine. Cortical spreading depression and aura may occur in the absence of headache, classified as typical aura without headache. Proponents of CSD believe that all migraines involve CSD and that silent auras occur in areas of the cortex in which changes in activity are not easily perceptible. Conversely, others argue that aura is an incidental phenomenon found in a minority of headaches.

Sex differences in trigeminal pain disorders

A characteristic feature of migraine, as well as temporomandibular disorder, is a marked sex disparity. Migraine and TMD are both more prevalent in women (Locker and Slade, 1988; Dworkin et al., 1990; Lipton et al., 1993; Stewart et al., 1994; Yunus, 2002). Women experience facial pain primarily in the years between menarche and menopause. Both migraine and temporomandibular disorder commonly begin around the time of menarche, increases during the reproductive years, and regress after menopause (LeResche et al., 2005). Although boys and girls have an equal incidence of migraine before puberty, the post-pubertal incidence increases in women and remains stable in men (Bille, 1997). A similar pattern is observed for temporomandibular pain. Despite the high prevalence of TMD and the disproportionate morbidity in women, the prevalance of TMD prior to puberty is low and equal between boys and girls (LeResche et al., 1997). The frequency of migraine episodes decreases after menopause following the decline and stabilization of ovarian hormones (Neri et al., 1993). Temporomandibular pain also decreases after menopause, and prevelence of TMD is lower for post-menopausal women than women of reproductive age (Carlsson and LeResche, 1995).

Menstrual cycle association with pain

Episodes of trigeminal pain are temporally related to the hormonal fluctuations of the menstrual cycle. Migraine attacks peak around the time of menstruation (Welch, 1997; Stewart et al., 2000) and more than 50% of female

migraineurs experience headache associated with menstruation (Couturier et al., 2003). Menstrual migraine pain is described as more severe than non-menstrual migraine by patients (MacGregor, 2000), and is of longer duration and more refractory to treatment (Couturier et al., 2003). Migraine without aura is associated with menstruation (common migraine). Although migraine with aura is not associated with menstruation (Russell et al., 1996), migraine with aura has been reported to be associated with high levels of estrogen in estrogen replacement, pregnancy, and during the normal cycle (MacGregor, 1999). Pure menstrual migraine is defined by the International Headache Society as migraine without aura that occurs exclusively on day 1+/-2 of the menstrual cycle (first day of menstruation) and at no other time during the cycle. Migraine that is associated with menstruation and also occurs at other points in the cycle is termed menstrually-related migraine (Olesen, 2004). Like headache, temporomandibular pain varies over the course of the menstrual cycle and peaks at menstruation (LeResche et al., 2003). Interestingly, temporomandibular pain shows a second mid-cycle increase in pain, corresponding to ovulation (LeResche et al., 2003). This mid-cycle peak does not occur in women taking oral contraceptives, which eliminate ovulation (LeResche et al., 2003).

Although migraines associated with ovulation have not been demonstrated by a controlled clinical study, there is some evidence to suggest that they occur (Martin et al., 2007a).

Estrogen

Estrogen is implicated as the link between trigeminal pain and the menstrual cycle. Use of estrogen containing oral contraceptives is associated with increased headache severity (Kudrow, 1975; Granella et al., 1993). Temporomandibular pain is worse in women taking oral contraceptives (LeResche, 1997) and those with high circulating estrogen (Landi et al., 2005). In post-menopausal women, estrogen replacement increases migraine incidence, which varies directly with the estrogen dose (Misakian et al., 2003). Estrogen replacement increases the frequency and severity of migraine, as well as analgesic use during episodes of pain (Martin et al., 2003). The effect of hormone supplementation on headache is sufficiently common for the International Headache Society to specify the term exogenous hormone-induced headache, which is the diagnosis for women regularly taking exogenous hormones who experience new onset of headache or increased frequency or severity of headache or for whom headaches remit with cessation of exogenous hormones (Olesen, 2004).

The estrogen paradox

Estrogen withdrawal has long been implicated as the precipitating factor for migraine associated with menstruation (Somerville, 1972). In women who experience regular menstrual migraine, preventing the late luteal estrogen decline with estrogen supplementation delays the migraine attack until estrogen is withdrawn, at which point migraine attack occurs (de Lignieres et al., 1986; Dennerstein et al., 1988). Migraine commonly occurs during the estrogen free

interval in women taking cyclical estrogen regimens (Kudrow, 1975). MacGregor and colleagues observed that migraine bears an inverse relationship to urinary estradiol across the menstrual cycle, and that attacks are more likely to occur during the late luteal phase, in which estrogen drops, than in the follicular phase, in which it rises (MacGregor et al., 2006). In 30-40% of pregnancies, headache occurs during the early post-partum period, during which estrogen levels fall (Stein, 1981).

Although these studies suggest that estrogen is protective, the data are complicated. High estrogen levels have been shown to induce headache (MacGregor, 1999) *and* estrogen exposure is a precondition for migraine with falling estrogen (Somerville, 1972). Moreover, a protective role of estrogen is inconsistent with fact that the majority of migraineurs are female.

Sex differences in experimental pain models

Studies of experimental pain models suggest that females are more sensitive to trigeminal nociception than males. Female rats show greater activity of Aδ and C trigeminal afferents than males in response to glutamate injection (Cairns et al., 2002). Intradermal capsaicin injection produces greater area and intensity of pain, as well increased brush allodynia in females compared to males (Gazerani et al., 2005). Similarly, female patients report greater intensity, duration, and area of pain than males following glutamate injection into the masseter, suggesting that the same effect occurs in humans (Cairns et al., 2001).

Properties of the trigeminal system change during the estrous cycle. Electrophysiological studies have demonstrated increased excitability of the trigeminal system during the high estrogen, proestrus phase of the estrous cycle.

There is also central nervous system plasticity during the estrous cycle since cutaneous receptive fields of central neurons in the spinal trigeminal nucleus increase in size during the proestrus phase of the rat estrous cycle (Bereiter and Barker, 1980). Responses to noxious stimuli are also sensitive to the phase of the estrous cycle. During proestrus, spinal trigeminal units increase their receptive field sizes and become more responsive to stimulation with bradykinin, suggesting that estrogen affects sensitivity of the trigeminal system *in vivo* (Okamoto et al., 2003). Intact cycling rats in proestrus and estrus phases have larger increases in receptive fields, and greater rate and magnitude of neuronal responses to capsaicin compared to rats in metestrus and diestrus (Martin et al., 2007b). Female animals also have increased central activation in response to inflammation, as mustard oil injection of the temporomandibular joint produces increased c-fos immunoreactivity in the nucleus caudalis in proestrus females compared to diestrus female, or male rats (Bereiter, 2001).

Differences in trigeminal pain according to sex and during the estrous cycle are related to the influence of estrogen on the trigeminal system. For example, estrogen treatment of ovariectomized rats increases the receptive field size and decreases the activation thresholds of neurons in the spinal trigeminal nucleus (Bereiter and Barker, 1975). Temporomandibular joint afferents are more responsive in females compared to males, and this effect is reduced by ovariectomy and restored by estrogen replacement (Cairns et al., 2002). Estradiol treatment of ovariectomized rats increases the spontaneous activity and decreases the activation thresholds of temporomandibular joint afferents *in vitro* (Flake et al., 2005).

While previous work has demonstrated sex differences in nociception, and changes in trigeminal function related to either sex or estrogen state, the effect of estrogen on trigeminal nociception has not been investigated using a behavioral model. Accomplishing this would bridge the gap between models that have demonstrated sex difference in nociception and models that show changes in trigeminal nerve function, showing changes in nociception with estrogen. This is one of the goals of the experiments in this dissertation.

While many studies suggest that estrogen has pro-nociceptive effects, the relationship between estrogen and pain is complex, and estrogen has anti-nociceptive effects in some models. Ovariectomized female mice display increased nociceptive behaviors following formalin injection of the upper lip compared to intact females (Multon et al., 2005). Similarly, aromatase knockout mice, which are unable to produce estrogen, show increased nociceptive responses to formalin injection compared to wild-type mice. This difference is eliminated by estrogen replacement, suggesting that estrogen is anti-nociceptive or that lack of estrogen is pro-nociceptive (Multon et al., 2005). Inflammation of the temporomandibular joint prolongs meal duration to a greater degree in males than in females (Bellinger et al., 2007), suggesting increased pain with mastication. Interestingly, models that shown pro-nociceptive effects of estrogen use formalin injection as a model of pain. Although formalin induces inflammation over the long term, it induces pain acutely through activation of receptors including TRPA1, a member of the transient receptor potential family of ion channels that is involved in sensing irritating chemicals. The anti-nociceptive effects in these models may be related to differential effects of

estrogen on the acute phase of pain present in the formalin model, compared to chronic inflammatory pain.

Estrogen may influence nociception through direct action on the trigeminal system. The trigeminal pathway, including the trigeminal ganglion is rich in estrogen receptors (Bereiter et al., 2005; Puri et al., 2005). The relationship between serum estrogen and ERα receptor expression is complex and appears to be highly dependent on both the estrogen level and tissue studied. For example, the number of ERα positive neurons is higher in females than in males throughout the trigeminal brainstem complex (Bereiter et al., 2005). In cycling animals, the number of ERα positive neurons surrounding the central canal of the trigeminal nucleus caudalis is greater in proestrus compared to diestrus (Bereiter et al., 2005), suggesting that short term increases in estrogen can upregulate ERα in the trigeminal system. Consistent with this concept, a single injection of estradiol increases the percentage of ERα labeled pelvic ganglion neurons projecting to the proximal urethra (Zoubina and Smith, 2003). Those changes may result from increased transcription of the estrogen receptor in the presence of estrogen or decreased turnover of ERα, as steroid receptor ligands have been shown to stabilize steroid receptors (Kemppainen et al., 1992). A goal of the experiments described in this dissertation is to investigate changes in receptor expression in the trigeminal ganglion with changes in the estrogen state.

The work in this dissertation focused on answering the following questions:

- How does estrogen affect inflammatory allodynia in the trigeminal system?
- Which estrogen receptors mediate these changes?

- How does the presence of estrogen and inflammation influence expression of estrogen receptors?

The Trigeminal Nerve

Headache and facial pain are conducted by the trigeminal nerve, cranial nerve V, which supplies primary sensory innervation to most cranial structures. The trigeminal nerve is composed of a three branch sensory root, which supplies sensory innervation to both superficial and deep structures of the face and anterior cranium and a motor root, which innervates the muscles of mastication. Cell bodies of the primary afferents are housed in the trigeminal ganglion, located in a recess of the middle cranial fossa known as Meckel's cave. The trigeminal ganglion gives rise to the three sensory branches—the ophthalmic (V1), maxillary (V2), and mandibular (V3) divisions. The ophthalmic branch passes through the superior orbital fissure and innervates the most superior structures of the head including the orbit, conjunctiva and cornea, upper eyelid, frontal sinuses, nasal mucosa, forehead and anterior scalp, as well as the meninges and the meningeal vasculature. The maxillary branch (V2) exits through the foramen rotundum and innervates more caudal structures including the lower eyelid, nares, upper cheek, maxillary, ethmoid and sphenoid sinuses, and upper palate, upper lip, and the maxillary teeth and gums. The mandibular branch projects through the foramen ovale and innervates the anterior 2/3 of the tongue, the mandible, lower lip and cheek, and the mandibular teeth and gums, anterior part of the external ear and part of the lateral scalp.

Trigeminal afferents enter the central nervous system at the level of the mid-pons. In the CNS, neurons that conduct information on discriminative touch project

to the principal sensory nucleus of V. Axons of neurons that conduct nociceptive information descend in the spinal trigeminal tract and synapse in the subnucleus caudalis in the spinal trigeminal nucleus. Axons of second order neurons in the nucleus caudalis decussate, ascend in the spinothalamic tract, and synapse on third-order neurons in the ventromedial thalamus.

The trigeminal nerve also includes a motor root that travels with the mandibular nerve and innervates the muscles of mastication—the masseter, temporalis, and medial and lateral pterygoid muscles—as well as the anterior belly of the digastric muscle, the mylohyoid muscle, the tensor veli palatini, and the tensor tympani. Cell bodies of the motor root fibers are located centrally in the pontine trigeminal motor nucleus. Neurons that receive proprioceptive information on jaw position and mechanoreceptive information from the teeth are also located in the CNS in the mesencephalic trigeminal nucleus.

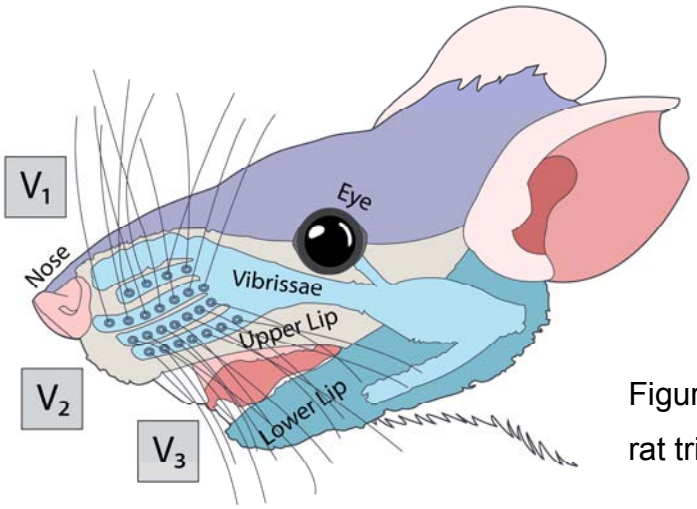

Figure I-1. Dermatomes of the rat trigeminal nerve

Cell types

Trigeminal ganglion neurons were historically classified as large light (A) and small dark (B) cells based on their histological appearance (Gaik and Farbman, 1973). Large light (A) cells correspond to neurons with thickly myelinated axons (Aα/β), while small dark cells (B) correspond to neurons with thinly myelinated or unmyelinated axons. Medium to large neurons have conduction velocities in the Aβ (14-30 m/s) or Aδ (2.2-8 m/s) range. Aβ are low-threshold mechanoreceptors and Aδ are either low-threshold mechanoreceptors, thermoreceptors, or nociceptors. Small C fiber neurons are considered to be nociceptors (Lawson, 1992). Glia, known as satellite cells, envelope the soma of neurons in the trigeminal ganglion (Pannese, 1981).

Neurons in the trigeminal ganglion are arranged somatotopically. Cell bodies of the mandibular division are located in the lateral and posterior ganglion, and cell

bodies of the maxillary and ophthalmic divisions are interspersed in the medial and anterior ganglion (Figure I-2). Although this relationship was described over two decades ago using retrograde tracing methods, the trigeminal ganglion has more recently been mapped using electrophysiological recording techniques (Leiser and Moxon, 2006). This work described the anatomy of the ganglion in sufficient detail to allow identification of the cell bodies that project to each of the three branches. Neurons innervating division 3 can easily be distinguished from those innervating divisions 1 and 2.

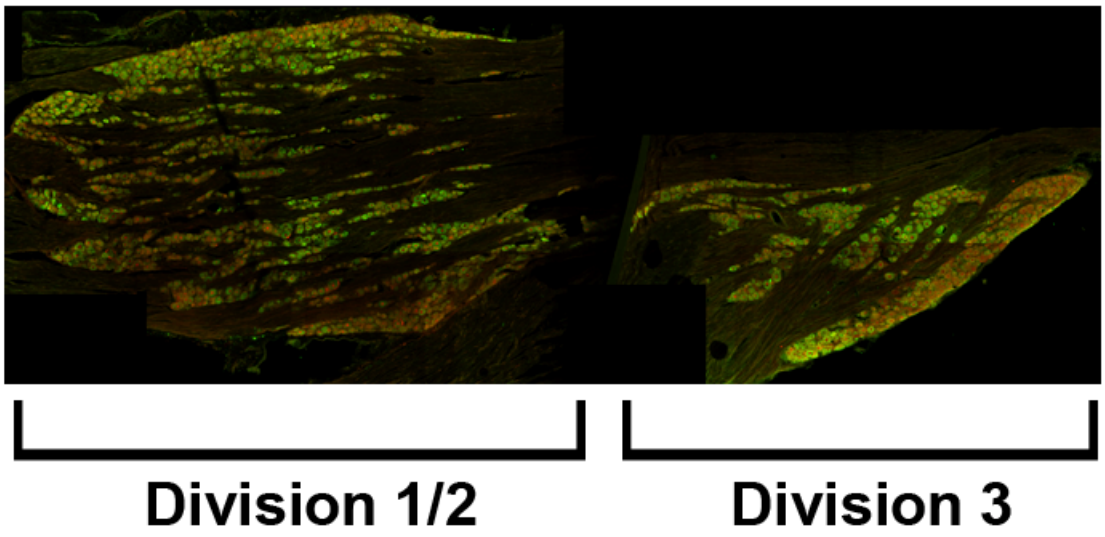

Figure I-2. Somatotopic arrangement of the trigeminal ganglion. Montage of 20x photomicrographs shows an entire trigeminal ganglion in horizontal cross section labeled with pERK (red) and TRPV1 (green). Divisions 1 and 2 are interspersed in the rostral and medial portion of the ganglion (left and top, respectively), and division 3 is located laterally and caudally.

A two-way street

First order trigeminal nociceptors are pseudounipolar, with axons that project bidirectionally to both peripheral tissues and the spinal trigeminal nucleus. In addition to transmitting afferent information from the periphery to the central nervous system, trigeminal neurons can also conduct action potentials in an anterograde direction, toward the periphery. Those anterograde action potentials can trigger the release of neurotransmitters from the peripheral terminals of trigeminal processes (Cuello et al., 1978). Numerous neurotransmitters and neuropeptides are expressed by neurons of the trigeminal ganglion, including CGRP, Substance P, serotonin, and neuropeptide Y (reviewed in Lazarov, 2000). Release of these transmitters can have inflammatory consequences in the periphery, inducing vasodilation, plasma extravasation, and degranulation of mast cells, a process referred to as neurogenic inflammation.

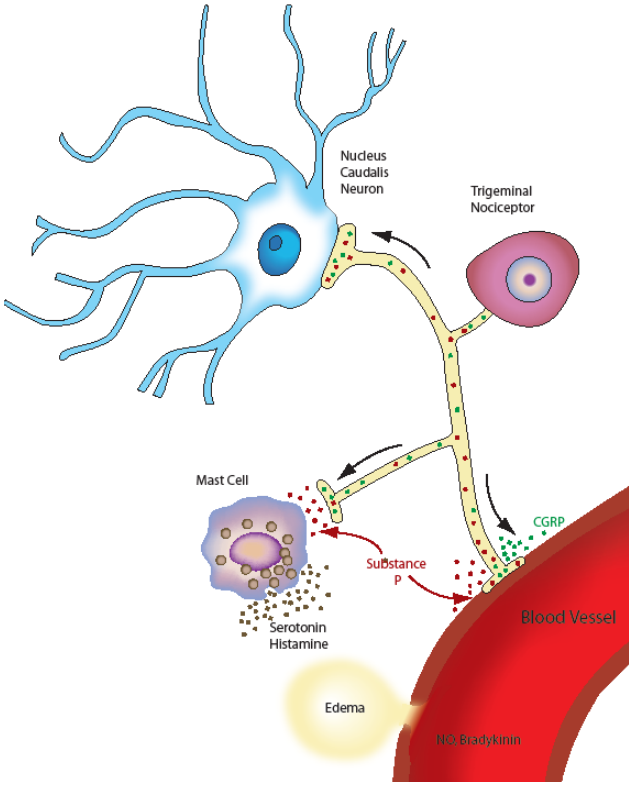

Figure I-3. Schematic of a first order trigeminal neuron. Anterograde conduction triggers release of mediators in the periphery leading to vasodilation, plama extravasation and mast cell degranulation.

Neurogenic inflammation can sensitize peripheral fibers and lead to enhanced conduction of nociceptive information to the spinal trigeminal nucleus (Strassman et al., 1996). Because the trigeminal ganglion, unlike the dorsal root ganglion, contains neurons that project to three separate dermatomes in close proximity, intracellular communication may lead to activation across divisions. Although it was previously believed that no axo-somatic synapses are present in the trigeminal ganglion, more recent evidence suggests that soma can communicate through paracrine release of neuropeptides or through gap junctions (Matsuka et al.,

2001; Thalakoti et al., 2007) and that satellite cells may facilitate communication even across ganglionic divisions.

Sensitization in migraine

Sensitization of the trigeminal system is involved in migraine and other disorders of trigeminal pain. Trigeminal sensitization is evident in the development of cutaneous allodynia, or the perception of an innocuous stimulus, such as a soft brush, as painful. Cutaneous allodynia occurs during attacks in the vast majority of migraineurs (Burstein et al., 2000a; Burstein et al., 2000b).

Sensitization during a migraine is thought to begin at the first order trigeminal afferent neuron (Levy et al., 2006), mediated by neurogenic inflammation. According to this hypothesis, a stimulus activates trigeminal meningeal afferents and triggers the release of neuropeptides that induce vasodilation, plasma extravasation, and inflammation, leading to sensitization of trigeminal afferents and migraine pain. The theory that neurogenic inflammation causes headache was first proposed by Wolff and Chapman more than fifty years ago, based on the observation that 'headache stuff', subcutaneous aspirates taken from headache patients, could induce vasodilation and pain when re-injected at distant sites (Wolff et al., 1953; Chapman et al., 1960). The concept that neurotransmitters are released from trigeminal nerve terminals and mediate sensitization in headache, was proposed by Moskowitz and colleagues (1979), and has subsequently been supported by a number of experimental studies. For example, electrical stimulation of the trigeminal ganglion increases cerebral bloodflow (Goadsby and Duckworth, 1987) and induces plasma extravasation in the dura, suggesting that trigeminal activation induces a neurogenic

inflammatory response (Markowitz et al., 1987). Dural plasma extravasation is reduced by neonatal capsaicin treatment, which destroys nociceptors, indicating that those neurons mediate neurogenic inflammation (Markowitz et al., 1987). Stimulation of the ganglion releases CGRP and substance P into the cerebral circulation (Goadsby et al., 1988), neuropeptides that can mediate neurogenic inflammatory processes. For example, intravenous infusion of substance P and CGRP induces dural plasma extravasation vasodilation, respectively (Markowitz et al., 1987). It is also known that chemical stimulation of the dura lowers mechanical thresholds of dural afferents (Strassman et al., 1996), suggesting that products of neurogenic inflammation can sensitize trigeminal neurons. It has been proposed that sensitized trigeminal afferents innervating the meningeal vasculature are activated with the pulse, leading to the throbbing character of migraine (Strassman et al., 1996).

Clinical evidence supports the neurogenic inflammation hypothesis. Serum CGRP levels are high during migraine episodes, suggesting the occurrence of a neurogenic inflammatory process (Goadsby and Edvinsson, 1993). A study using single photon emission computerized tomography (SPECT) to image patients experiencing migraine attacks showed accumulation of technetium 99 labeled albumin in the meninges, suggesting that menigeal plasma extravasation occurs during the course of a migraine (Pappagallo et al., 1999). Intracranial plasma extravasation during migraine has also been imaged using MRI and occurs in anatomical correspondence with the headache focus (Knotkova and Pappagallo, 2007). Notably, all effective anti-migraine therapies to date have been shown to inhibit neurogenic inflammation in animal models (Johnson and Bolay, 2006). Triptans are the current standard of treatment for migraine. The first triptan on the

pharmaceutical market was sumatriptan. Sold as Imitrex and Imigran, sumatriptan is administered to elevate low serotonin levels present during the onset of migraine. Triptans function as 5-HT (types 5-HT1B and 5-HT1D) agonists. 5-HT receptors are found on cells in the cranial and basilar arteries. Activation of those receptors results in their activation and vasoconstriction of the dilated arteries. Sumatriptan has also been postulated to abort migraine attacks through reduction in CGRP release, as sumatriptan reduces CGRP release from trigeminal neurons and reduces plasma CGRP during migraine (Goadsby and Edvinsson, 1993; Durham et al., 2006).

Peripheral sensitization of primary afferents may lead to central sensitization. According to the theory of central sensitization, input from sensitized first-order trigeminal afferents sensitizes second-order neurons in the trigeminal nucleus caudalis. This lowers the nociceptive threshold for sensory pathways that converging on these sensitized second-order neurons. Chemical stimulation of dural afferents enhances the response of second order sensory neurons in the trigeminal nucleus caudalis to stimulation of facial skin, taken to indicate central sensitization in the trigeminal system (Burstein et al., 1998). Central sensitization has been postulated to explain the common occurrence of allodynia that spreads across dermatomal boundaries, referred to as secondary allodynia (Burstein and Jakubowski, 2004). Central sensitization has also been proposed to mark the close of the temporal window for when triptan therapy is effective (Burstein et al., 2004).

Although sensitization in migraine is well documented, the events that initiate sensitization in migraine are not well defined. According to one view, championed by Moskowitz and colleagues, migraine is a disorder of cortical excitability that activates trigeminal afferents, leading to headache. This hypothesis centers on cortical

spreading depression, a wave of cortical depolarization (CSD), followed by a wave of hyperpolarization, first described by Leao (1944). Alterations in cortical blood flow accompany CSD, and a wave of hyperemia followed by a wave of oligemia, or decreased perfusion. Cortical spreading depression has been demonstrated during the course of migraine (Olesen et al., 1981; Cao et al., 1999; Hadjikhani et al., 2001). A link between neurogenic inflammation and CSD was provided by Bolay and colleagues who demonstrated that CSD causes dural vasodilation, plasma extravasation and c-fos activation in the trigeminal nucleus (Bolay et al., 2002). All of those measures were attenuated by transection of the trigeminal nerve, suggesting that CSD induces neurogenic inflammation by activating dural trigeminal afferents (Bolay et al., 2002). Cortical spreading depression is thought to activate trigeminal neurons by triggering release of substances from neurons and glia, such as potassium, nitric oxide, protons, and glutamate (Moskowitz, 2007).

Critics of the CSD hypothesis have cited key inconsistencies in CSD as the initiating event in migraine, the foremost being that at least 70% of migraineurs do not experience aura. Conversely, aura commonly occurs without headache, and abortion of the aura does not affect the headache course, suggesting that aura may be a parallel process unrelated to the headache itself (Goadsby, 2001).

While research on trigeminal sensitization has examined processes in the trigeminal braimstem nuclei and peripheral terminals, considerably less attention has been paid to potential mechanisms in the ganglion itself. The trigeminal ganglion is a likely site of sensitization, as it is the central structure for peripheral and central theories. Additionally, it is important to note that trigeminal sensitization also occurs in temporomandibular disorders, which affect a similar demographic population as

migraine, suggesting that a cortical source of trigeminal sensitization is unlikely, as these disorders are not accompanied by aura. The goal of the studies described in this dissertation was to investigate sensitization of the trigeminal ganglion with inflammation and estrogen, and whether intervention at the ganglion itself affects sensitization.

Estrogen receptors

Estrogen receptors α and β

Estrogen receptor α (ERα) is a member of the steroid/thyroid superfamily of nuclear receptors and functions as a ligand-dependent transcription factor that modulates gene expression. ERα contains three major functional domains: a sequence-specific DNA-binding domain that recognizes estrogen response elements (EREs) in the promoter regions of target genes; a ligand binding domain; and an activator function domain that mediates protein-protein interactions. Upon binding estrogen, estrogen receptor α subunits dimerize, allowing interaction with transcription factors and estrogen response elements in DNA and ultimately producing complex effects on gene expression (Nilsson et al., 2001). Downstream effects of these classical 'genomic' actions of ERα require synthesis of new RNA and protein, and occur over a period of hours. Although it was believed for a number of years that estrogen's effects are mediated by a single receptor, the characterization of estrogen receptor β (ERβ) over a decade ago proved otherwise (Kuiper et al., 1996). ERβ is a nuclear receptor that is similar in structure and function to ERα. ERβ has a DNA binding domain that is highly homologous to ERα (Enmark et al.,

1997) but differs in the activator function domain. Consequently, although ERβ binds to similar genetic elements, its responses to ligands differ from ERα. For example, partial agonists of estrogen receptor α such as tamoxifen are pure antagonists of estrogen receptor β (Barkem, et al., 1998).

The rapid signaling estrogen receptor

There are longstanding data confirming that estrogen induces nuclear effects over a matter of hours, however, cellular responses to estrogen also occur rapidly in a matter of minutes through activation of signal transduction pathways. "Nongenomic" effects were first described in 1977 (Pietras and Szego, 1977), but research on those pathways lagged behind studies of nuclear actions for many years. The effects of estrogen on signal transduction pathways are known to be important in many physiological processes including cardiovascular protection, bone preservation, cancer cell proliferation, and neuroprotection.

There is evidence that non-genomic activity of estrogen can be mediated by ERα. Estradiol has been shown to activate second messenger pathways, including the MAPK kinase ERK, in cell lines that express ERα, but not in ERα-negative cells (Pedram et al., 2006). Transfection of cell lines with ERα confers the ability to activate ERK in response to estradiol treatment (Acconcia et al., 2005b). Development of the ERα selective agonist PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol) has facilitated identification of ERα -specific effects. PPT is a potent agonist that binds ERα with a 410-fold higher binding affinity than ERβ (Stauffer et al., 2000). PPT treatment rapidly activates signal transduction

mediators in several cell types, providing further evidence for ERα-mediated signal transduction (Klinge et al., 2005).

Non-genomic estrogen effects are thought to be mediated by a form of ERα that is sequestered to the cytoplasm or the plasma membrane within a signaling complex (Manavathi and Kumar, 2006). The initial evidence for this concept was provided by studies showing that signal transduction is initiated by estradiol conjugated to large, membrane impermeant molecules such as bovine serum albumin. For example, rapid ERK activation occurs in response to estradiol-BSA in cells that contain membrane-localized ERα (Chen et al., 2004). Several mechanisms have been implicated in targeting ERα to cytoplasmic signaling complexes. Some evidence suggests that post-translational modifications play a role. For example, palmitoylation of ERα has been shown to be necessary for ERK activation in ERα transfected cells (Acconcia et al., 2004). A variety of proteins have also been implicated in targeting ERα to signaling complexes. ERα associates with caveolin 1 and 2 which target the receptor to the plasma membrane and allow association with a signaling complex (Razandi et al., 2002). Metastatic tumor antigen 1 (MTA1) sequesters estrogen receptor to the cytoplasm and enhances non-genomic signaling in breast cancer cells (Manavathi et al., 2007). Although it is clear that ERα participates in signal transduction in many systems, whether ERα participates in signal transduction in trigeminal neurons has not been investigated.

GPR30

An alternative mechanism for rapid signaling by estrogen that has generated a great deal of interest is GPR30, a G-protein coupled receptor. G protein-coupled

receptors (GPRs) transduce their signals via G-protein heterotrimers that dissociate into free G-subunit protein and G-subunit protein complexes after stimulation with the appropriate ligand. GPR30 was first described in estrogen responsive breast adenocarcinoma cell lines (Carmeci et al., 1997), and was known as an orphan receptor until recently when it was characterized as a novel estrogen receptor that operates exclusively through signal transduction mechanisms (Revankar et al., 2005; Thomas et al., 2005; Funakoshi et al., 2006). Actions of GPR30 can be blocked *in vitro* by pertussis toxin (Filardo et al., 2000), which blocks most receptor-G-protein interactions. Tumor cells that lack both ERα and ERβ but express GPR30 bind estrogen with high affinity (Filardo et al., 2000). GPR30 is expressed at high levels in aggressive cancer cells and at much lower levels in nonagressive cells, suggesting an involvement of GPR30 in growth of hormone-sensitive tumors (Carmeci et al., 1997).

GPR30 mediates estrogen effects in many cell types. In endometrial carcinoma cells, G-protein inhibitors and GPR30 antisense oligonucleotide (Vivacqua et al., 2006a) attenuate *c-fos* expression induced by estradiol and tamoxifen, an ERα antagonist. GPR30 functions in proliferation of several types of tumor cells (Vivacqua et al., 2006b; Vivacqua et al., 2006a; Albanito et al., 2007).

G-proteins are usually located at the plasma membrane, however, there are conflicting data regarding the subcellular location of GPR30. GPR30 is present in the cytoplasm of neurons in the rat hypothalamus (Brailoiu et al., 2007; Sakamoto et al., 2007) and localizes to the endoplasmic reticulum in GPR30-transfected COS cells (Revankar et al., 2005), in which only cell-permeable estrogens activate downstream signaling, suggesting that the intracellular form of GPR30 is functional

(Revankar et al., 2007). By contrast, other studies suggest that GPR30 is localized to the plasma membrane like other, previously characterized G-protein coupled receptors. Studies of estrogen binding to GPR30 suggest that the receptor is localized to the cell surface in breast cancer cells (Thomas et al., 2005). Confocal analysis of GPR30-expressing hippocampal neurons showed localization of GPR30 on the plasma membrane (Funakoshi et al., 2006). GPR30 may traffic between the cytoplasm and membrane, as suggested by the translocation of GPR30 to the cytoplasm in response to estradiol stimulation of hippocampal neurons (Funakoshi et al., 2006).

One of the targets of 'non-genomic' estrogen actions is the MAP kinase ERK. Estrogen rapidly activates ERK in neuronal cells, including neuroblastoma cells (Watters et al., 1997), cerebral cortical explants (Setalo et al., 2002), cultured DRG (Purves-Tyson and Keast, 2004) and trigeminal neurons (Puri et al., 2006). The receptor through which ERK activation occurs in trigeminal neurons has not been determined. There is considerable precedent for the activation of ERK by both ERα and GPR30. Transfection of cells with the GPR30 gene confers the ability of estradiol to activate ERK, and estradiol has been shown to activate ERK in cells that express GPR30, but not ERα or ERβ (Filardo et al., 2000). GPR30-mediated ERK activation promotes proliferation of ovarian, endometrial, and thyroid tumor cells (Vivacqua et al., 2006b; Vivacqua et al., 2006a; Albanito et al., 2007). Signaling through ERα may also activate ERK. The ERα agonist PPT activates MAPK in vascular endothelial cells (Klinge et al., 2005), and ERα transfection allows ERK to be activated by estrogen (Acconcia et al., 2005a). Signaling through either receptor

has been demonstrated in the same cell type, as selective agonists for either ERα or GPR30 stimulate proliferation of ovarian carcinoma cells (Albanito et al., 2007).

With the expansion of knowledge of estrogen signaling and receptors there are still many questions to be answered about estrogen and trigeminal pain. Trigeminal neurons express ERα, but little ERβ (Bereiter et al., 2005). Whether GPR30 is present in the trigeminal system and which estrogen receptor functions in trigeminal sensitization are investigated in the experiments described in this dissertation.

ERK

The mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that mediate cellular responses to a wide variety of signals, including hormones, cytokines, and growth factors (Widmann et al., 1999). One member of this family is extracellular-signal regulated kinase, or ERK, also known as p44/42 MAPK. ERK mediates a multitude of cellular responses, and is widely studied as a promoter of cell division. The catalytic activity of ERK is activated by dual phosphorylation at serine and threonine residues by the upstream kinase MAPK/ERK Kinase (MEK). Upon activation, ERK phosphorylates downstream targets in both the nucleus and cytoplasm, including the transcription factors CREB, elk-1, and c-myc (Sweatt, 2001).

ERK has been traditionally viewed as a cell division kinase, however, it is also a significant mediator in nociceptive pathways. Ji and colleagues (Ji et al., 1999) showed ERK to be a specific marker of nociceptive input that is activated in the dorsal horn in a dose dependent manner by noxious stimuli. Subsequent work

has examined ERK activation in nociceptive pathways using pain models. ERK is activated in dorsal root ganglion following nerve injury (Doya et al., 2005), hindpaw inflammation (Obata et al., 2003), and by joint movement in the setting of inflammatory arthritis (Seino et al., 2006). Similarly, ERK is activated in the dorsal horn by nociceptive stimuli such as pin prick and noxious heat (Ji et al., 1999). Following spinal nerve ligation, ERK activation occurs sequentially in dorsal horn neurons, microglia, and astrocytes (Zhuang et al., 2005). These observations have led to the widespread use of ERK as a pain marker.

In addition to being a specific marker of nociceptive input, ERK activation has been demonstrated to be mechanistically involved in the development of hyperalgesia (increased sensitivity to pain) in models of both inflammatory and neuropathic pain. The selective MEK inhibitor U0126 suppresses mechanical and thermal allodynia following chronic constriction of the sciatic nerve, inflammation, or formalin injection of the rat hindpaw (Obata et al., 2003) Using a transgenic mouse in which dominant negative MEK is expressed exclusively in neurons, Gereau and colleagues demonstrated reduced pain behaviors and thermal hyperalgesia following formalin injection, suggesting that ERK activation in neurons is important for hyperalgesia (Karim et al., 2006).

Although the role of ERK activation in pain has been studied extensively in the spinal cord and dorsal root ganglion, there is relatively little work on ERK in trigeminal pathways. Existing evidence suggests a similar function of ERK in the trigeminal system, as ERK is activated in the trigeminal nucleus caudalis by passive jaw movement in the presence of TMJ inflammation (Suzuki et al., 2007) and by capsaicin injection of the tooth pulp (Shimizu et al., 2006).

ERK mechanisms

The mechanism of ERK-mediated pain has not been clarified, and may involve cytoplasmic and transcriptional downstream events. Several molecular mechanisms of ERK-mediated hyperalgesia have been proposed. ERK has been shown to increase neuronal excitability through phosphorylation of the Kv4.2 subunit of the voltage-gated A-type potassium channel (Adams et al., 2000; Hu and Gereau, 2003). In Kv4.2 knockout mice, ERK-dependent forms of pain hypersensitivity are absent (Hu et al., 2006). Nociception mediated by the vanilloid receptor, TRPV1 may be mediated by ERK, as activated ERK is necessary for facilitation of heat-induced TRPV1 currents in dorsal root ganglion neurons (Firner et al., 2006). Genes downstream of ERK may also function in its pro-nociceptive effects. For example, antagonizing ERK reduces expression of TRPV1 and NK-1 (Ji et al., 2002a; Obata et al., 2004). In trigeminal neurons, ERK has been shown to promote expression of the vasodilatory neuropeptide CGRP, which is implicated in the pathogenesis of migraine, suggesting a possible link between ERK and trigeminal pain disorders (Durham and Russo, 1998).

Estrogen treatment activates ERK in primary cultures of dorsal root ganglion (Purves-Tyson and Keast, 2004) and trigeminal ganglion (Puri et al., 2006). Given the relationship between ERK activation and nociception, this poses the question of whether estrogen contributes to trigeminal pain through ERK activation. The estrogen receptor that mediates ERK activation in trigeminal neurons has not been investigated. In other systems, ERK may be activated by either ERα or GPR30.

The goal of this dissertation was to investigate whether ERK activation functions in estrogen-modified trigeminal nociception. Although estrogen activates ERK in sensory neuron cultures, whether ERK is activated by estrogen in trigeminal ganglion *in vivo* has not been determined. Furthermore, the receptors that mediate ERK activation in trigeminal neurons are unknown and are investigated in the studies described in this dissertation.

CFA as a model of inflammatory pain

Inflammation of peripheral tissues is a component of trigeminal pain disorders. During migraine, neurogenic inflammation results in plasma extravasation and the release of inflammatory mediators such as leukotrienes, cytokines, prostaglandins, and histamine from mast cell granules. In order to understand the responses of sensory neurons to inflammatory conditions, it is useful to create a simulated inflammatory milieu in animal models.

A commonly used model of peripheral inflammation in animals is injection of Complete Freund's Adjuvant (CFA). CFA is a suspension of heat-killed *Mycobacterium tuberculosis* in paraffin oil that was developed for use in boosting the antibody response to vaccines. CFA is not approved for medical use in humans due to the painful inflammatory response it induces, which was bravely described by Gould (2000), using himself as an experimental subject (Gould, 2000). A loss to medicine was nonetheless a gain to the field of pain research, as CFA proved to be an effective model of inflammatory pain. CFA, as a depot preparation that remains confined to the tissue into which it was injected, produces localized inflammation. It induces a stable inflammatory response over a period of days, allowing pain and its

modulators to be assessed over time. Plasma extravasation occurs and pro-inflammatory cytokines are produced at the site of inflammation (Watanabe et al., 2005; Ambalavanar et al., 2006b; Flake et al., 2006). CFA does not induce systemic inflammation, however, as intra-muscular CFA does not increase pro-inflammatory cytokines in serum (Watanabe et al., 2005). Because of these advantages, CFA has become a well established pain model used to induce inflammation in a variety of sites, including orofacial structures.

CFA-mediated inflammation increases nociceptive responses to both thermal and mechanical stimuli (Ji et al., 2002b), and induces both primary hyperalgesia at the site of inflammation, and secondary hyperalgesia distant from the site of inflammation. These properties have been demonstrated in two models of orofacial pain, CFA injection to the temporomandibular joint capsule (Flake et al., 2005; Takeda et al., 2005b) and intra-muscular injection into the masseter (Ambalavanar et al., 2006a). CFA administered to either the temporomandibular joint or the masseter lowers the withdrawal threshold for mechanical stimulation (Sessle and Hu, 1991; Takeda et al., 2004) and induces secondary mechanical hyperalgesia of the whisker pad (Takeda et al., 2005b).

CFA-induced inflammation increases excitability of nociceptive pathways. Inflammation of the temporomandibular joint increases excitability to facial mechanical stimulation *in vivo* (Takeda et al., 2006), and the fraction of TMJ-afferents that respond to capsaicin (Takeda et al., 2005a). A previous report showed increased excitability of TMJ afferent neurons when rats were concurrently administered estrogen replacement. Although peripheral inflammation has been demonstrated to induce trigeminal sensitization using behavioral models, and

estrogen has been shown to modulate excitability of the trigeminal system using electrophysiological approaches, the effect of estrogen on inflammatory nociception in the trigeminal system has not been investigated using a behavioral model. In this dissertation we use CFA as an inflammatory agent in order to determine the effect of estrogen replacement on trigeminal sensitization induced by peripheral inflammation.

Assessing trigeminal nociception

Although the effect of estrogen on the excitability of trigeminal afferents has been evaluated using single unit recording, a useful next step is to investigate the effect on nociception. Nociception in animal studies is ideally assessed using behavioral models, and many methods have been developed. Nociceptive assays measure spontaneous pain behaviors, thermal nociception, or mechanical nociception. For studying trigeminal pain, it is ideal to assess mechanical sensitivity, as patients with trigeminal pain disorders exhibit hypersensitivity of that modality. The most established method for assessing mechanical nociception is the use of graded monofilaments, which deliver a reproducible mechanical force, allowing treatment groups to be compared with respect to either withdrawal threshold or withdrawal frequency. Although monofilament testing has been successfully employed in assessing inflammatory and neuropathic pain in the rodent hindpaw (Gandhi et al., 2004), those techniques prove difficult to translate to the facial region. One of the principal challenges of orofacial testing is that it is necessary to keep the rodent's head stationary in order to reliably test mechanical sensitivity—a moving rat makes it exceedingly difficult to reproducibly test the same area, to be confident that

a constant stimulus is being delivered, and to discern withdrawal responses from spontaneous movements. Although mechanical testing of the orofacial region using graded monofilaments has been described in previous reports (Ren, 1999; Ambalavanar et al., 2006a) that method has a significant disadvantage in that it involves handling of the animal by the investigator during testing. Handling of experimental subjects alters the behavioral response to stimuli and has been shown to induce experimental bias (Chesler et al., 2002), which may explain the large withdrawal thresholds (>50g) observed in those models. One of the goals of this project was to develop a reliable and sensitive method for assessing mechanical hyperalgesia in the facial region. In this dissertation, we employ a new model for assessing the effect of estrogen on nociception.

Specific Aims of the project

Migraine is a highly prevalent and severely disabling disorder. Although migraine episodes are closely associated with estrogen, the mechanisms linking the two are poorly understood. This dissertation project had three related aims:

- Although estrogen increases excitability of trigeminal afferents, the relationship between estrogen and trigeminal nociception, as measured using a behavioral model, has remained unclear. The first goal of this project was to develop a behavioral model for assessing mechanical sensitivity of the orofacial region and to use this model to assess the *in vivo* effects of estrogen on inflammatory allodynia.

- The molecular events linking estrogen and nociception are poorly understood. The MAP kinase ERK is a key mediator of inflammatory pain and is activated by estrogen in sensory neurons *in vitro*. The second goal of this project was to investigate whether ERK activation plays a role in estrogen-modified nociception.

- The biological significance of non-genomic signaling mediated by estrogen receptors has become increasingly clear, particularly with the recent discovery of the novel estrogen receptor GPR30. The third goal of this project was to determine which estrogen receptors in the trigeminal ganglion function in ERK activation and estrogen-modified nociception.

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II. Estrogen increases nociception through ERK activation in the trigeminal ganglion: Evidence for a peripheral mechanism of allodynia

Abstract

Migraine is much more common in women than in men, and painful episodes are linked to the hormonal fluctuations of the menstrual cycle. Migraineurs often present with allodynia in the distribution of all three branches of the trigeminal nerve (V1, V2, and V3), which is assumed to be centrally mediated. Because trigeminal ganglion neurons express estrogen receptors, estrogen may act peripherally to modulate pain responses. We used an established model of inflammatory trigeminal pain, injection of Complete Freund's adjuvant (CFA) in the masseter muscle, to study the effect of estrogen treatment on inflammatory primary and secondary allodynia/hyperalgesia. Ovariectomized female Sprague-Dawley rats were injected in the masseter with CFA or saline and subcutaneously administered estradiol valerate or vehicle. Withdrawal response to stimulation of the masseter (V3, primary) and whisker pad (V2, secondary) using von Frey filaments was measured. Masseter inflammation, both with and without estrogen, increased withdrawal to masseter stimulation 24 and 48 hours following injection. Estrogen treatment in the presence of masseter inflammation increased withdrawal response to masseter stimulation at 48 and 72 hours compared to inflammation alone. In order to assess secondary hyperalgesia, mechanical sensitivity of the whisker pad was measured 24, 48 and 72 hours following injections. Masseter inflammation increased withdrawal response to whisker pad stimulation 24 hours following injection. In conjunction with estrogen treatment inflammation increased withdrawal response compared to CFA alone, indicating an additive effect of inflammation and estrogen on secondary hyperalgesia/allodynia. We examined activation of extracellular-signal regulated kinase (ERK), a MAPK involved in inflammatory pain, in the trigeminal ganglion of each group using Western blot and

immunohistochemistry. Masseter inflammation or estrogen treatment increased ERK activation compared to vehicle. Masseter inflammation in conjunction with estrogen treatment increased ERK activation compared to inflammation alone.

In order to determine whether there is increased ERK activation in division 1/2, we compared the percentage of neurons in each group containing activated ERK relative to total neurons in division 1/2 and division 3. In division 1/2, either masseter inflammation or estrogen increased the percentage of pERK immun4oreactive neurons relative to vehicle-treated OVX controls, and concurrent masseter inflammation and estrogen treatment increased pERK immunoreactivity in V1/2 compared to inflammation or estrogen alone. In division 3, inflammation, both with and without estrogen, increased the percentage of pERK immunoreactive neurons compared to vehicle. Estrogen treatment increased pERK immunoreactivity relative to vehicle. We stereotactically administered ERK antagonist U0126, or inactive control U0124, to the trigeminal ganglion of CFA+E2-treated rats. Animals treated with U0126 had decreased withdrawal responses to von Frey filament stimulation of the whisker pad compared to U0124-treated rats. Because the secondary allodynia in V2 after inflammation in V3 was reduced by antagonizing ERK activation in the periphery, these data suggest a peripheral component to secondary inflammatory trigeminal allodynia/hyperalgesia mediated through activation of ERK.

Introduction

Trigeminal pain disorders, such as migraine and temporomandibular disorders (TMDs), are more prevalent in women than in men (Manzoni, 1995; Manzoni et al., 1995; Dworkin et al., 2002; Lyngberg et al., 2005), which has been attributed to estrogen fluctuations over the course of the menstrual cycle (Somerville, 1972). Episodes of trigeminal pain correlate temporally with the menstrual cycle, increasing at menarche (LeResche et al., 2005) and declining with cessation of the menstrual cycle after menopause and during pregnancy (Lipton and Bigal, 2005). Trigeminal pain in women changes with the menstrual cycle. Migraine increases during the perimenstrual period (MacGregor and Hackshaw, 2004), and TMJ pain increases mid-cycle (LeResche et al., 2003) and at menstruation. Headache and TMDs share common features (Glaros et al., 2007), and both may be accompanied by significant allodynia (Burstein et al., 2000b; Sarlani and Greenspan, 2003). Although clinical data suggest that estrogen increases susceptibility to trigeminal pain disorders, the fall in estrogen at the time of menstruation may precipitate an attack, suggesting a complex relationship between estrogen levels and trigeminal pain.

Exogenous estrogens have been shown to have pro-nociceptive effects. Estrogen replacement therapy decreases thermal pain thresholds (Fillingim and Edwards, 2001) and may trigger migraine with aura (MacGregor, 1999). Use of exogenous estrogens, such as hormone replacement therapy or oral contraceptives, increases the risk of TMD pain (LeResche et al., 1997). Sex differences in trigeminal pain disorders affecting different cranial sites suggests that the basis for their sex disparity

lies in the trigeminal system. It has been suggested that these effects result from direct actions of estrogen on the trigeminal system, which is rich in estrogen receptors (Bereiter et al., 2005). In animal studies, cutaneous receptive fields of neurons in the spinal trigeminal nucleus expand in phase with estrogen levels during the rodent estrous cycle and with estrogen replacement following ovariectomy (Bereiter and Barker, 1975, 1980). Estrogen replacement *in vivo* has been shown to increase the excitability of acutely dissociated primary trigeminal afferents (Flake and Gold, 2005). However, it has not been determined whether these changes in trigeminal excitability translate to allodynia in the awake animal.

The mechanism through which estrogen modulates excitability of trigeminal neurons is unclear. A possible mediator is activation of extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family. Activation of ERK occurs through a dual phosphorylation event mediated by the upstream kinase MAPK/ERK kinase (MEK). In sensory pathways, ERK phosphorylation is a specific marker of nociceptive activation (Ji et al., 1999) and is required for thermal and mechanical hyperalgesia in several models of peripheral nerve injury and inflammation (Obata et al., 2004b, a). Estrogen has been shown to activate ERK in cultured neurons from dorsal root ganglion (DRG) (Purves-Tyson and Keast, 2004) and trigeminal ganglion (Puri et al., 2006), but the effects of *in vivo* estrogen on ERK activation in sensory ganglion has not been examined.

Cutaneous allodynia is common in trigeminal pain disorders (Burstein et al., 2000a; Burstein et al., 2000b; Sarlani and Greenspan, 2003) and is an indicator of trigeminal sensitization that may be assessed using graded mechanical stimuli. In the current

study, we used a behavioral model of trigeminal inflammatory pain to determine whether estrogen modulates mechanical allodynia. We also investigated the *in vivo* effects of estrogen treatment on ERK phosphorylation and the role of ERK activation in the development of orofacial allodynia following inflammation and estrogen treatment.

The results of this study support the hypothesis that estrogen-mediated ERK activation in the trigeminal ganglion modulates responses to peripheral inflammation, and suggest that facial allodynia is not a solely central phenomenon, but is also mediated, in part, by peripheral sensitization of the trigeminal ganglion.

Methods

Animals: 60-75 day old virgin female Sprague-Dawley rats (Harlan, Indianapolis, IN) were used for all studies. Rats were maintained on a daily 12 hour light, 12 hour dark schedule with *ad libitum* access to water and food (Harlan Teklad 8604). All studies were conducted in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals and the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Ovariectomy: Rats were anesthetized with 4% isoflurane in compressed oxygen and maintained on 1% isoflurane. Dorsal bilateral incisions were made midway between the lower ribs and the iliac crest, and both ovaries were isolated and removed. The incision was closed with suture clips and Buprenex was administered (0.1mg/kg). Rats were allowed to regain consciousness before returning to the animal facility.

CFA treatment and hormone replacement:

Two weeks following ovariectomy, rats were given a single 50 µl injection of a Complete Freund's Adjuvant (CFA; Mycobacterium tuberculosis H37 Ra; Sigma, St. Louis, MO) solution, 1:1 in 0.1M phosphate buffer into the left masseter muscle under isoflurane anesthesia. We used 50 µl of 1:1 CFA solution, a dose that has been shown to produce molecular and behavioral effects that last for 3 days (Ro, 2005; Lee and Ro, 2007). Control rats were injected with an equal volume of saline. Estradiol valerate (E2, Delestrogen, Monarch Pharmaceuticals, Bristol, TN), an estradiol conjugate that provides prolonged stable estrogen levels (Oriowo et al., 1980) 10 µg/kg in sesame oil (100µl), used as a vehicle, was injected subcutaneously using a 22 gauge needle during the same surgery as masseter injection. All control rats received sesame oil vehicle. Post-mortem examination of CFA-treated rats and hematoxylin and eosin staining of sectioned facial structures revealed granulomatous inflammation confined to the masseter muscle. We did not observe inflammatory pathology upon examination of cranial sites outside the masseter, including the whisker pad. This is supported by other studies using the CFA model that demonstrate inflammation confined to the injection site (Ogawa et al., 2003; Takeda et al., 2005b; Ambalavanar et al., 2006b).

Orofacial sensitivity assay: The behavior apparatus is shown in Figure II-1. Rats were conditioned to traverse a clear plastic tunnel attached to a behavioral arena (A in Figure II-1) in order to gain access to a bottle containing 0.1M sucrose (C in Figure II-1). In this task, the rat must extend its head from a hole in the end of the tunnel in

order to sample drops of sucrose solution. During the drinking task, the rat voluntarily held its head in a fixed position, allowing easy and precise access to specific areas of the face for mechanical stimulation. Rats were acclimated to the apparatus for 15 minute periods once daily for 5 days. During each session, the rats consume approximately 2 mL sucrose, less than 10% of their total daily water intake. This amount of sucrose consumption is less than 1% of the amount required to produce antinociceptive effects in adult rats, approximately 1M sucrose *ad libitum* over three to four weeks (Kanarek et al., 2001), and acute effects of sucrose on analgesia, present in neonates, disappear by P21 (Anseloni et al., 2002). The rats were not restrained in any way, and the tunnel is large enough for the rat to turn around to reenter the arena at any time. After training, they spent approximately ¼ of their time in the testing position when placed in the behavioral arena. Once rats were fully trained and reliably demonstrated acquisition of target behavior, withdrawal responses to orofacial mechanical stimulation were assessed using graded monofilaments (Stoelting, Wood Dale, IL), a well established method for assessing mechanical sensitization in rodents. Rats were coded and experimenters blinded to experimental groups. The behavioral responses were scored as follows: no response (scored 0); flinch/orienting reflex (scored 1); partial retraction (scored 2); or complete retraction (scored 3). A flinch/orienting reflex was classified as a discontinuation in licking behaviors and orientation toward the stimulus. A partial retraction was classified as retraction of the head away from the reward. Escape back into the tunnel or arena was scored as a full retraction. A percent withdrawal score was calculated for each rat as the sum of response scores divided by the total possible score, a score of 3 on all trials. Withdrawal was assessed in response to stimulation of the whisker pad or skin overlying the masseter using 0.16 g and 4 g

monofilaments, respectively. Preliminary studies established that these filaments produced withdrawal responses at or below threshold in control rats. Mechanical withdrawal responses were measured 24, 48, and 72 hours following CFA and estradiol treatments.

Hormone measurements: Blood (1.5 ml) was collected in microcentrifuge tubes by cardiac puncture at the time of perfusion. Blood was centrifuged at 7000x g for 5 min, and the plasma transferred into fresh tubes. Serum estradiol levels were measured by radioimmunoassay according to the method described in Terranova and Garza (Terranova and Garza, 1983). Mean estradiol values were 37 ± 8 pg/ml for estrogen-replaced animals, which corresponds to a proestrus level (Martin and Behbehani, 2006) and 4 ± 2 pg/ml for ovariectomized vehicle treated animals.

Western blot: Trigeminal ganglia ipsilateral to the injection site were harvested and homogenized in cold lysis buffer (20 mM Hepes buffer, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 mg/ml pepstatin A, 10 mg/ml leupeptin and 10 mg/ml aprotinin) using a Dounce homogenizer. The homogenate was centrifuged at 12,000 g (10 min, 4 °C) and the supernatant was collected. Total protein concentration was determined with a bicinchoninic acid (BCA) assay kit using bovine serum albumin as a standard (Pierce Biotechnology, Inc., Rockford, IL). Proteins were separated using SDS-PAGE on 12%TRIS-HCl gels (BioRad, Hercules, CA) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were simultaneously probed with rabbit antibody specific for the dually-phosphorylated, active form of ERK (p-ERK) (1:200, Cell Signaling Technology, Beverly, MA) and goat anti-glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were rinsed at room temperature in TBS-Tween-20 and IRDye 800 conjugated anti-goat (Rockland Immunochemicals, Gilbertsville, PA) and Alexa 680 conjugated anti-rabbit (Invitrogen, Eugene, OR) secondary antibodies were applied 1:10,000 in blocking buffer for 1 hour at room temperature. Membranes were rinsed at room temperature in Tris-buffered saline containing Tween-20 (TBST) followed by TBS and visualized using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). For total ERK, membranes were stripped for 30 minutes in a solution of 62.5 mM Tris HCl, 2% SDS, and 100mM β-mercaptoethanol and reprobed using a mouse monoclonal antibody to total ERK (1:1000, #4696, Cell Signaling Technology, Beverly, MA) followed by an Alexa 680 conjugated goat anti-mouse secondary antibody (1:10,000, Molecular Probes). Band intensities were determined using Odyssey software version 1.0 (LI-COR, Lincoln, NE) and expressed as a ratio of pERK/total ERK.

Immunohistochemistry: Rats were deeply anesthetized with sodium pentobarbital (50mg/kg i.p.) and perfused transcardially with 0.1M PBS followed by 4% buffered paraformaldehyde. Trigeminal ganglia were removed, post-fixed overnight in 4% buffered paraformaldehyde and cryoprotected in 30% sucrose in 0.1 M phosphate buffer pH 7.2. Ganglia were frozen in Tissue Freezing Medium (Triangle Biomedical Science, Durham, NC) at -80° C. Frozen sections (20μm) through the horizontal plane of the trigeminal ganglion were prepared using a cryostat (Carl Zeiss, Inc., Oberkochen, Germany). Horizontal sectioning of the ganglion allows visualization of the three anatomic divisions in a single section. The sections were permeabilized and blocked for 1 hour in a solution of 0.3% Triton-X100 and 2% normal goat serum

in 0.1M phosphate buffered saline (PBS). Activated ERK was labeled by incubating overnight at 4°C in an antibody specific for the active form of ERK, dually-phosphorylated at Thr202/Tyr204 of ERK1 and Thr185/Tyr187 of ERK2 (1:200, #4376, Cell Signaling Technology, Beverly, MA), followed by 1 hour in AlexaFluor 568 goat anti-rabbit (1:500, Molecular Probes, Carlsbad, CA). Some slides were co-labeled using guinea pig anti-TRPV1 (1:1000, Neuromics, Edina, MN) and AlexaFluor 488 goat anti-guinea pig (Molecular Probes). Slides were coverslipped using ProLong Gold anti-fade reagent with DAPI (Molecular Probes).

Microscopy and analysis: Fluorescent digital images were obtained on a Nikon Eclipse 90i upright microscope and Nikon C1 confocal imaging system using a 20x objective and a frame size of 1024 × 1024 pixels. Sections stained with the primary antibody omitted were used to control for non-specific fluorescence. Detector gain and laser intensity were set initially using control slides, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter. This ensured that all neurons considered immunoreactive in images of stained sections had fluorescence intensities above background. Individual images were montaged into a single image using Adobe Photoshop CS2 to prevent double counting on the image edges and to allow localization of immunoreactivity to subdivisions. In preliminary experiments, all neuronal profiles were immunoreactive for total ERK. Neuronal profiles positive for activated ERK and total profiles per section were counted from montaged photomicrographs in a blinded fashion. Neuronal cells were differentiated from glia by their distinctive large, round morphology and pale DAPI staining nuclei. A minimum of 2000 neurons were

counted from at least 2 randomly selected sections from each of 6 animals per treatment condition.

Stereotactic injection: Rats were fixed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) under isoflurane anesthesia. The trigeminal ganglion was targeted by stereotaxic coordinates (Schneider et al., 1981) and the injection carried out according to the method described by Whitehead et al. (Whitehead et al., 2003). A midline incision was made in the scalp and a 1mm burr hole was drilled in the left calvarium at 2.5mm lateral and 3 mm posterior to bregma. A 27-gauge Hamilton syringe was lowered slowly into the trigeminal ganglion. Because central structures of the trigeminal pathway are located contralaterally at this level, stereotactic injection ipsilateral to testing avoids structures of the tested sensory pathway. Correct placement of the needle was confirmed by observing the masseter twitch upon the needle entering the trigeminal ganglion. The MEK inhibitor U0126 or its inactive congener U0124 (Tocris Bioscience, Ellisville, MO) 100 μM in 10% DMSO (5 μl) was injected over 5 minutes. The scalp was closed with suture clips. Rats were administered Buprenex (0.1mg/kg) and allowed to recover before returning to the animal facility.

Statistical analysis: Data were analyzed using Graph Pad Prism version 4.0 software (Graph Pad, San Diego, CA). Behavioral data and immunohistochemical data were analyzed by one-way ANOVA using a Bonferroni post hoc test. Data are presented as mean ± SEM, with a P value less than 0.05 considered significant.

Results

Estrogen and inflammation additively increase primary and secondary allodynia

In order to determine how estrogen influences mechanical nociception in the presence of orofacial inflammation, we used Complete Freund's Adjuvant (CFA) to induce inflammation of the masseter. CFA injection is an established model of inflammatory pain (Ji et al., 2002; Obata et al., 2004a; Duric and McCarson, 2007) that can induce widespread mechanical hyperalgesia (Ambalavanar et al., 2006a). In order to assess facial mechanical sensitivity, we developed a behavioral method based on operant conditioning that allows monofilament testing of the territory innervated by the trigeminal nerve (Figure II-1). Using this method, the 50% withdrawal threshold for stimulation of the masseter in preliminary experiments was 4g. This model has advantages over other models of mechanical testing of the orofacial region that require direct contact between the subject and experimenter (Ren, 1999) and result in higher baseline withdrawal thresholds, perhaps due to handling (Chesler et al., 2002).

We conducted a preliminary study to determine the monofilament force resulting in a 50% withdrawal threshold in ovariectomized rats. A sub-threshold hair was subsequently used for the testing of whisker pad and masseter in order to measure allodynia. In order to assess primary allodynia, we stimulated the masseter using a 4g monofilament (Figure II-2A). Masseter inflammation, both with and without estrogen, increased withdrawal responses to stimulation of the skin overlying the masseter 24 and 48 hours following injection ($p<0.05$). Estrogen treatment in the

presence of masseter inflammation increased withdrawal responses at 48 and 72 hours compared to inflammation alone ($p<0.05$).

In order to assess secondary allodynia, mechanical sensitivity of the whisker pad was measured using a 0.16g monofilament 24, 48 and 72 hours following CFA injection (Figure II-2B). Masseter inflammation increased withdrawal response to whisker pad stimulation 24 hours following injection ($p<0.05$ compared to vehicle, one-way ANOVA). Estrogen treatment combined with masseter inflammation increased withdrawal response to whisker pad stimulation at 24 hours compared to inflammation alone ($p<0.05$) and at 48 hours compared to vehicle ($p<0.05$).

ERK is activated in trigeminal ganglion by inflammation and estrogen

Because ERK, an established mediator of inflammatory nociception, is activated by estrogen in trigeminal ganglion neurons maintained in culture (Puri et al., 2006), we explored whether ERK activation contributed to the development of allodynia in our behavioral model. We first examined ERK activation at 24 hours, when CFA-treated rats demonstrated mechanical allodynia in the behavioral assay.

We used Western blots to analyze ERK activation in trigeminal ganglion samples from rats treated with CFA+E2, CFA, E2, and vehicle-treated rats. Membranes labeled with an antibody specific for the dually-phosphorylated, activated form of ERK showed two bands of molecular weight 44 and 42 kD, corresponding to the phosphorylated forms of ERK1 and ERK2, respectively. A representative blot of trigeminal ganglion pERK is shown in Figure II-3A. Either masseter inflammation or

estrogen treatment increased ERK1 and ERK2 activation, compared to vehicle (Figure II-3B). Masseter inflammation in conjunction with estrogen treatment increased ERK activation compared to inflammation alone ($p<0.05$).

In order to examine the anatomical distribution of ERK activation, immunohistochemically labeled frozen sections of trigeminal ganglion from each treatment group—CFA+E2, CFA, E2, and vehicle were compared to determine the percentage of neuronal profiles containing activated ERK relative to total neuronal profiles per ganglion. In preliminary experiments, ganglion sections labeled with an antibody to total ERK showed immunoreactivity in all neurons. Activated ERK was present in the nuclei and cytoplasm of neuronal soma.

The somatotopic arrangement of the trigeminal ganglion has long been established, but recent work has clarified the anatomy of the ganglion to allow identification of cell bodies projecting to each of the three branches (Leiser and Moxon, 2006). Neurons innervating division 3 can easily be distinguished from those innervating divisions 1 and 2 based on anatomic markers (Figure II-4). The neurons with axons innervating the masseter, that are exposed to local inflammation following CFA treatment, are located in division 3, while the whisker pad is innervated by division 2. The allodynia of the whisker pad following inflammation of the masseter suggests that neurons located in division 2 may have increased excitability. In order to determine whether there is increased ERK activation in each division, we compared the change in proportion of neurons containing activated ERK in division 1/2 and division 3. In division 1/2, either masseter inflammation or estrogen increased the percentage of pERK immunoreactive neurons relative to vehicle-treated OVX controls ($p<0.05$),

and concurrent masseter inflammation and estrogen treatment increased pERK immunoreactivity in V1/2 compared to inflammation or estrogen alone (Figure II-6). In division 3, inflammation, both with and without estrogen, increased the percentage of pERK immunoreactive neurons compared to vehicle ($p<0.05$, Figure II-6). Estrogen treatment increased pERK immunoreactivity relative to vehicle ($p<0.05$).

Blockade of ERK activation reduces secondary allodynia.

In order to determine whether ERK activation within the ganglion is required for trigeminal sensitization, the effect of peripheral administration of an inhibitor of ERK activation on allodynia was determined using our behavioral model. We administered U0126 using stereotactic injection, an established method for targeting agents to the trigeminal ganglion (Whitehead et al., 2003; Karai et al., 2004). Twenty-fours hours after treatment with CFA and E2, a timepoint following the development of allodynia, we stereotactically injected the MEK inhibitor U0126, or its inactive congener U0124, into the trigeminal ganglion ipsilateral to CFA treatment. We assessed withdrawal responses 24 hours following administration of U0126/4. U0126 treatment reduced the withdrawal response to whisker pad stimulation compared to U0124-treatment ($p<0.05$, Figure II-7).

Discussion:

The results of this study provide evidence that estrogen increases the duration of primary allodynia and both the duration and magnitude of secondary allodynia in the region innervated by the trigeminal nerve in a model of trigeminal inflammatory pain.

Data also show that inflammation of the masseter increases neuronal ERK activation in all three divisions of the trigeminal ganglion, and that estrogen increases ERK activation in the trigeminal ganglion in both the presence and absence of peripheral inflammation. Furthermore, the results demonstrate that locally blocking ERK activation in the trigeminal ganglion reduces secondary allodynia as shown by increased mechanical sensitivity of whisker pad after CFA induced inflammation of the masseter. The results of this study strongly suggest that ERK activation in the trigeminal ganglion is important for maintenance of facial allodynia, and that estrogen amplifies nociceptive activation of ERK.

Estrogen exacerbates inflammatory trigeminal nociception

The behavioral results show that estrogen increases the duration of primary inflammatory allodynia of the facial region. This is the first study to demonstrate enhanced sensitization of the trigeminal territory by estrogen in a behavioral model of allodynia. Estrogen has previously been shown to alter the physiological properties of trigeminal neurons. High estrogen states, in both normal cycling and estrogen replacement, increase receptive field sizes in the spinal trigeminal nucleus (Bereiter and Barker, 1975, 1980). The current results are consistent with the data of Flake, *et al*. (Flake et al., 2005) who demonstrated increased excitability of dissociated trigeminal afferents three days following estrogen treatment and inflammation of the temporomandibular joint. By extending this work to a behavioral model, the results of this study suggest that previously described increases in excitability and receptive field size result in hyperalgesia in the awake animal.

The effect of estrogen on secondary allodynia was tested using mechanical stimulation of the whisker pad following inflammation of the masseter, a measure of sensitization extending across dermatomal divisions. Results show that inflammation of the masseter increases withdrawal responses to whisker pad stimulation, indicating that inflammation of the mandibular branch can sensitize the maxillary branch. Secondary hyperalgesia following peripheral inflammation has been demonstrated previously. Widespread sensitization occurs following CFA inflammation (Ambalavanar et al., 2006a), and inflammation of the TMJ decreases escape threshold and increases excitability of whisker pad afferents in response to mechanical stimulation (Takeda et al., 2005b).

Results show that inflammatory secondary sensitization is increased by the addition of estrogen. While estrogen increased the duration of allodynia to primary site stimulation, it increased both the duration and magnitude of sensitization of the whisker pad. Estrogen thus appears to spatially broaden the area of allodynia. These results may be relevant to cutaneous allodynia, a common clinical manifestation of secondary hyperalgesia that occurs in a large proportion of migraineurs and TMD patients (Sarlani and Greenspan, 2003; Burstein and Jakubowski, 2004). Cutaneous allodynia varies with the menstrual cycle, and the allodynic area is greater in women during both the luteal and menstrual phases of the cycle compared to men (Gazerani et al., 2005). The current findings suggest mechanisms by which estrogen could exacerbate cutaneous allodynia during episodes of migraine and TMD.

ERK is activated by estrogen in the trigeminal ganglion

Results show that inflammation of the masseter increases ERK activation in neurons of the trigeminal ganglion. ERK is specifically activated by nociceptive input (Ji et al., 1999), and neuronal ERK activation mediates hyperalgesia (Karim et al., 2006). ERK is activated by peripheral inflammation in dorsal root ganglion and the spinal cord dorsal horn (Doya et al., 2005). In the trigeminal system, ERK is activated in the nucleus caudalis by passive jaw movement in the presence of TMJ inflammation (Suzuki et al., 2007) and by capsaicin injection of the tooth pulp (Shimizu et al., 2006).

Our data provide evidence that estrogen treatment alone can activate ERK in sensory ganglia of female rats. Estrogen can have direct effects on trigeminal ganglion neurons, as estrogen receptor α is prevalent throughout the trigeminal pathway, including neurons of the trigeminal ganglion (Bereiter et al., 2005; Puri et al., 2006). ERK activation in the presence of increased estrogen *in vivo* is potentially significant in light of the demonstrated role of ERK in mechanisms of pain and provides a potential mechanism linking high estrogen states and increases in excitability of trigeminal ganglion neurons.

Furthermore, these data show that estrogen administered in the presence of masseter inflammation produces a larger increase in ERK activation than inflammation alone. An additive effect of estrogen on ERK activation in the context of inflammation may have important implications for trigeminal pain. Neurogenic inflammation mediated by peripheral terminals is implicated as an important

mechanism of migraine and other disorders of trigeminal pain *(Waeber and Moskowitz, 2005).* Our results suggest that, during trigeminal pain episodes, ERK activation in the trigeminal ganglion induced by peripheral inflammation would be increased by a surge in estrogen. Additional ERK activation induced by estrogen may sensitize trigeminal neurons to the degree that a subacute inflammatory stimulus becomes painful.

Analysis of ERK activation in the maxillary/ophthalmic division (1/2) shows that masseter inflammation increases ERK activation outside the third division of the trigeminal ganglion. This is an intriguing finding, as it shows that peripheral inflammation can activate ERK in soma that have no direct contact with the inflamed tissue. These results suggest that ERK activation can occur through intercellular communication within the ganglion. It is possible that neurons containing activated ERK can activate the soma of neighboring cells through paracrine signaling. Neuropeptides, including CGRP (Ulrich-Lai et al., 2001), are released within the trigeminal ganglion from neuronal soma, and may signal to neighboring cell bodies (Matsuka et al., 2001). TMJ inflammation has been shown to increase excitability of Aβ neurons projecting to the whisker pad via an NK1 mediated mechanism, presumably through local release of substance P (Takeda et al., 2005a). TNFα administration into the TMJ increases p38 MAPK activation throughout the trigeminal ganglion by neuron-glia communication through gap junctions (Thalakoti et al., 2007). Because the trigeminal ganglion, unlike the dorsal root ganglion, contains neurons that project to three separate dermatomes in close proximity, intracellular communication may lead to activation across divisions, contributing to trans-dermatomal allodynia, as seen in this study.

Peripheral ERK activation in trigeminal sensitization

Results of this study show an additive effect of estrogen and inflammation on ERK activation within the maxillary/ophthalmic division of the trigeminal ganglion. Because of the role of ERK in mediating somatic pain (Obata et al., 2003) and the current behavioral results showing that inflammation and estrogen sensitize the orofacial region in an additive manner, we asked whether ganglionic ERK activation is involved in changes in facial sensitivity. The data from these experiments provide evidence that delivery of an inhibitor of ERK activation to the trigeminal ganglion reduces facial sensitivity, and ERK activation mediates increased peripheral sensitization of trigeminal neurons. Whereas many studies have employed intrathecal infusion of MEK inhibitors, in this study, a MEK inhibitor was delivered directly to the ganglion in order to examine the role of peripheral activation in the primary afferents. The current results suggest that peripheral ERK activation within trigeminal ganglion neurons is an important component of sensitization of the orofacial region.

Cutaneous allodynia has been proposed to arise when input from sensitized first-order trigeminal afferents sensitizes second-order neurons in the trigeminal nucleus caudalis, thereby lowering the activation threshold of convergent first-order neurons, a phenomenon known as central sensitization. Chemical stimulation of dural afferents enhances the response of second order sensory neurons in the trigeminal nucleus caudalis to stimulation of facial skin, taken to indicate central sensitization in the trigeminal system (Burstein et al., 1998). The current observation that secondary

allodynia is ameliorated by administration of MEK inhibitor to the trigeminal ganglion suggests that ERK activation in neurons of the trigeminal ganglion contributes to the maintenance of cutaneous allodynia. These results are potentially significant to our understanding of the pathogenesis of cutaneous allodynia, as they suggest that processes within the trigeminal ganglion contribute to cutaneous allodynia. While there is undoubtedly a role for central sensitization in development of allodynia, the presence of allodynia across trigeminal dermatomes alone does not prove that central sensitization occurs, and, conversely, the existence of central sensitization does not preclude the contribution of intra-ganglionic mechanisms.

Several studies have shown reduction of pain-related behaviors by MEK inhibitors administered prior to or concurrent with injury. Our data show that U0126 reduced secondary allodynia when administered following the development of sensitization. Our results are similar to those of Seino and collegues who showed alleviation of pain by U0126 administered by intra-articular injection of the knee following development of inflammation in an arthritis model (Seino et al., 2006). The ability to modulate facial pain following development of allodynia has important implications for the treatment of migraine, since onset of allodynia signals the close of the temporal window during which triptan intervention is effective, ostensibly due to central sensitization (Burstein et al., 2004; Burstein and Jakubowski, 2004). Triptans are thought to function by acting on 5HT1B/D receptors in the trigeminal ganglion, and sumatriptan reduces CGRP release in cultured trigeminal ganglion neurons (Bellamy et al., 2006). An alternative argument for necessity of early triptan treatment is that triptans function by suppression of intra-ganglionic communication, and that allodynia signals closure of the window for preventing of cross sensitization

of ganglionic divisions. In that case, pharmacologic approaches targeting the periphery, such as ERK antagonism or reducing intraganglionic CGRP release, may be viable alternatives.

The results in this study suggest an intra-ganglionic component to facial allodynia. Intra-ganglionic sensitization may function in conjunction with central sensitization in the generalization of pain during the course of a trigeminal pain episode. Results suggest that estrogen increases secondary sensitization by broadening the sensitized area via ERK activation in the trigeminal ganglion. These findings strongly suggest the possibility that modulation of signal transduction pathways in the periphery following the development of central sensitization may be a useful avenue in the management of trigeminal pain.

Acknowledgments

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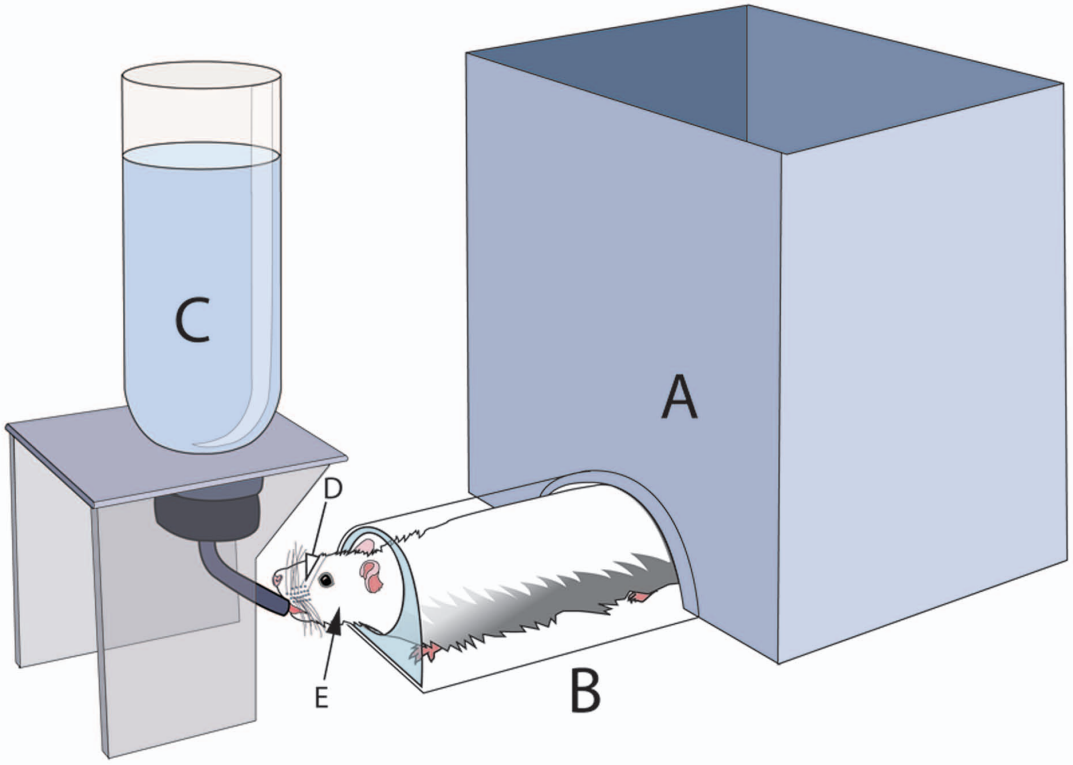

Figure II-1 Behavioral assay of orofacial sensitivity. Rats were placed in the behavioral arena (A) and conditioned to crawl from a through a clear plastic tunnel (B) in order to reach a bottle containing 0.1M sucrose (C). In this task, the rat must extend its head from a hole in the end of the tunnel in order to drink the sucrose solution. This places the rat's head in a fixed position, which allows easy and precise access to specific areas of the face for monofilament stimulation. Rats have free movement between the box and tunnel at all times. Once animals were fully trained and reliably demonstrate acquisition of target behavior, withdrawal responses to facial stimulation of the whisker pad (D) and masseter (E) were assessed using monofilaments.

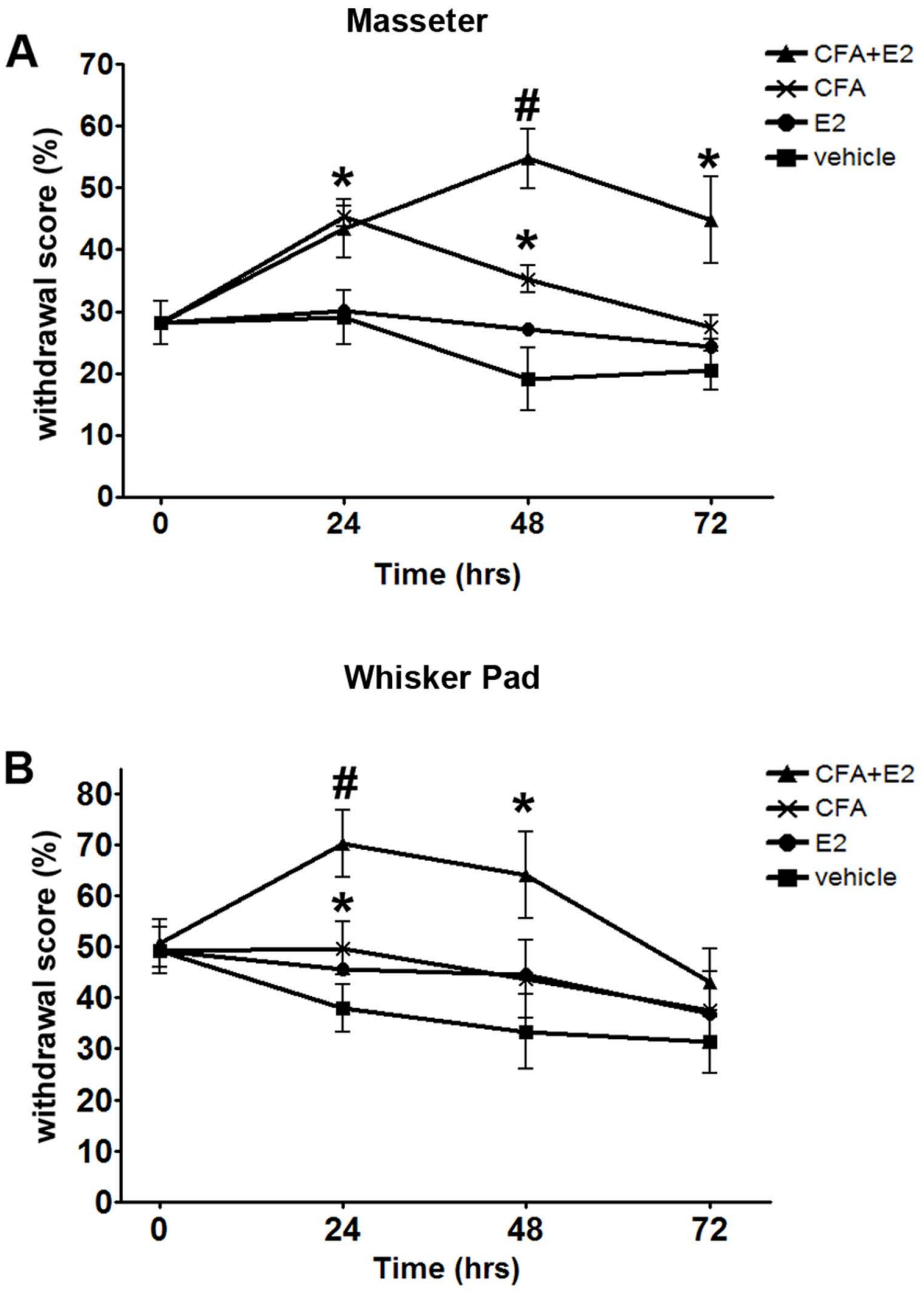

Figure II-2 Estrogen replacement increases primary and secondary facial hyperalgesia. **A** Primary allodynia. Withdrawal scores in response to monofilament (4g) stimulation of the masseter. **B** Secondary allodynia. Withdrawal scores in response to monofilament (0.16g) stimulation of the whisker pad. For each animal, the left masseter of was injected intra-muscularly with 50 μl CFA (1:1 in saline) or saline vehicle and estradiol valerate (E2,10 μg/kg in sesame oil) or sesame oil vehicle was administered subcutaneously. N=8-12 per group; *=$p<0.05$ compared to vehicle and #=$p<0.05$ compared to CFA, one-way ANOVA.

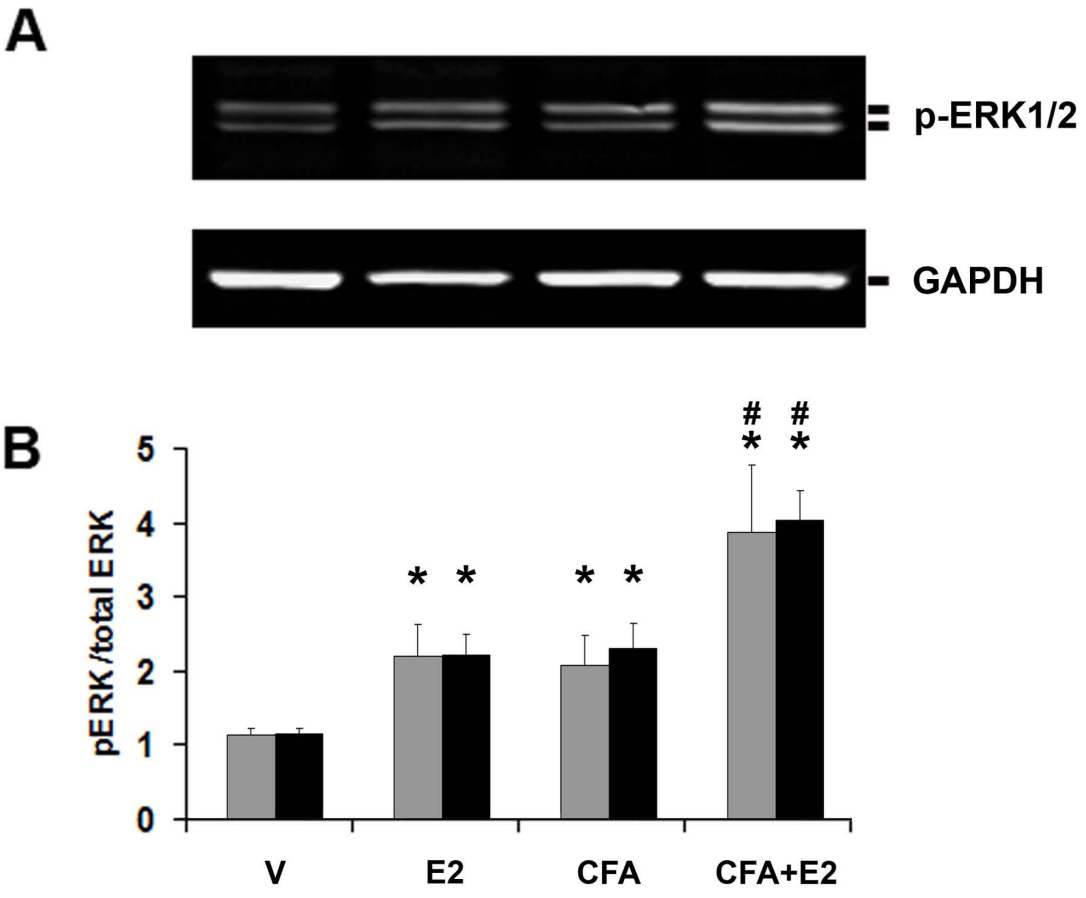

Figure II-3 Estrogen replacement combined with masseter inflammation increases ERK activation in trigeminal ganglion. **A)** Western blot of pERK in trigeminal ganglion from (left to right) vehicle, E2, CFA, and CFA+E2. Proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane and probed with a pERK specific antibody that recognizes two bands corresponding to the phosphorylated forms of p44 and p42. Blots were stipped and reprobed for total ERK to obtain pERK/total ERK for each sample. **B)** Fold change of mean integrated intensity of pERK/ERK relative to vehicle. Data are shown as mean+/- SEM. N=8 per group; * $p<0.05$ compared to vehicle and # $p<0.05$ compared to CFA, one-way ANOVA.

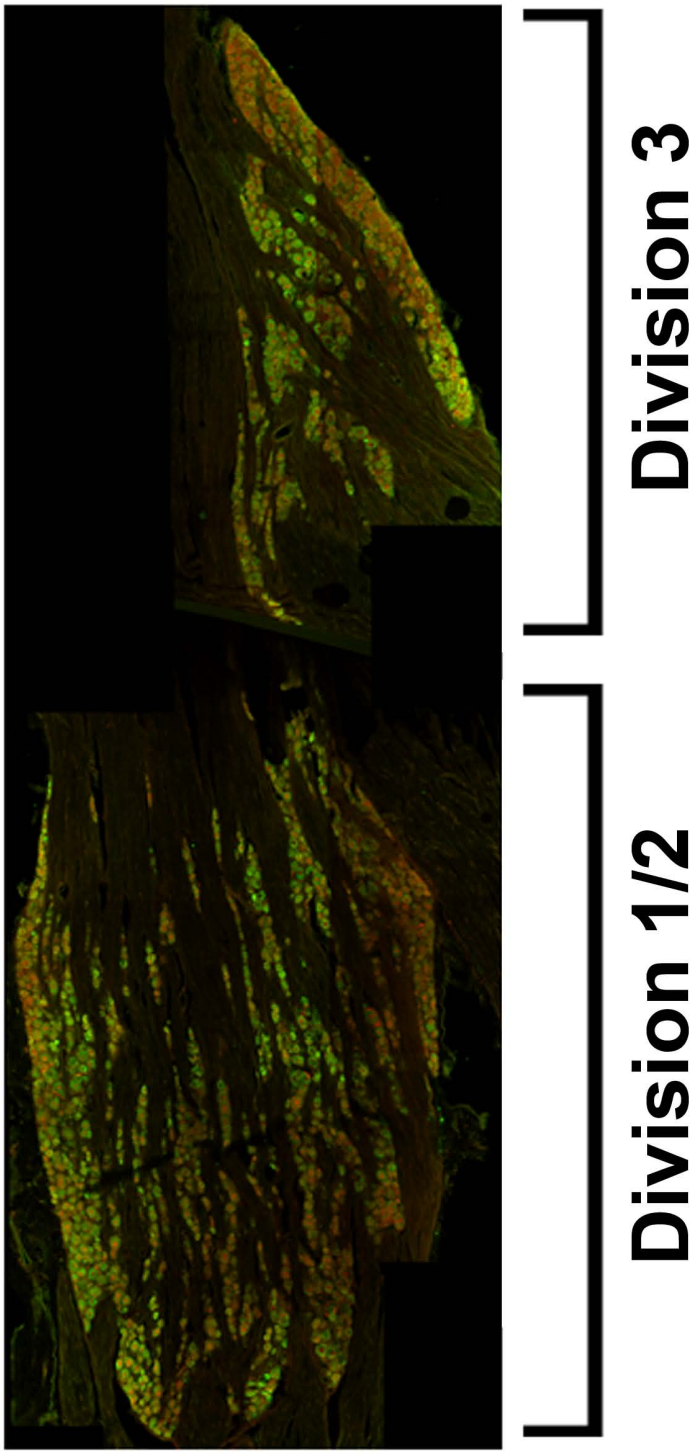

Figure II-4 Somatotopic arrangement of the trigeminal ganglion. Montage of 20x photomicrographs shows an entire trigeminal ganglion in horizontal cross section labeled with pERK (red) and TRPV1 (green). Divisions 1 and 2 are interspersed in the rostral and medial portion of the ganglion (left and top, respectively), and division 3 is located laterally and caudally.

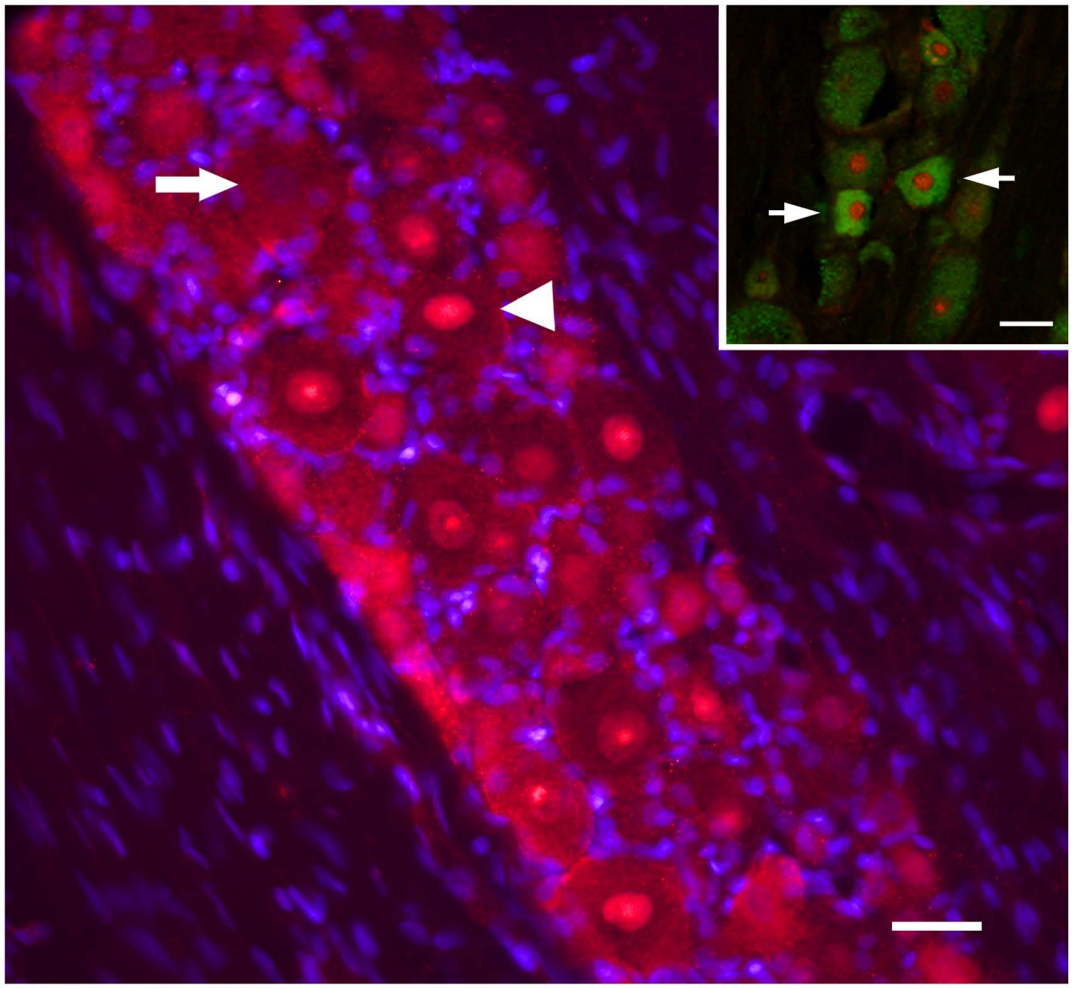

Figure II-5 ERK activation in the rat trigeminal ganglion. Immunohistochemistry for pERK (red) shows staining in neurons, which can be identified by their characteristic large, round morphology and pale blue DAPI staining nuclei. The arrowhead shows an ERK positive neuron, and an ERK negative neuron is indicated by the arrow. The inset shows double labeling of pERK (red) and the nociceptor marker TRPV1 (green). Arrows in the inset indicate co-localization. Scale bars = 30μm

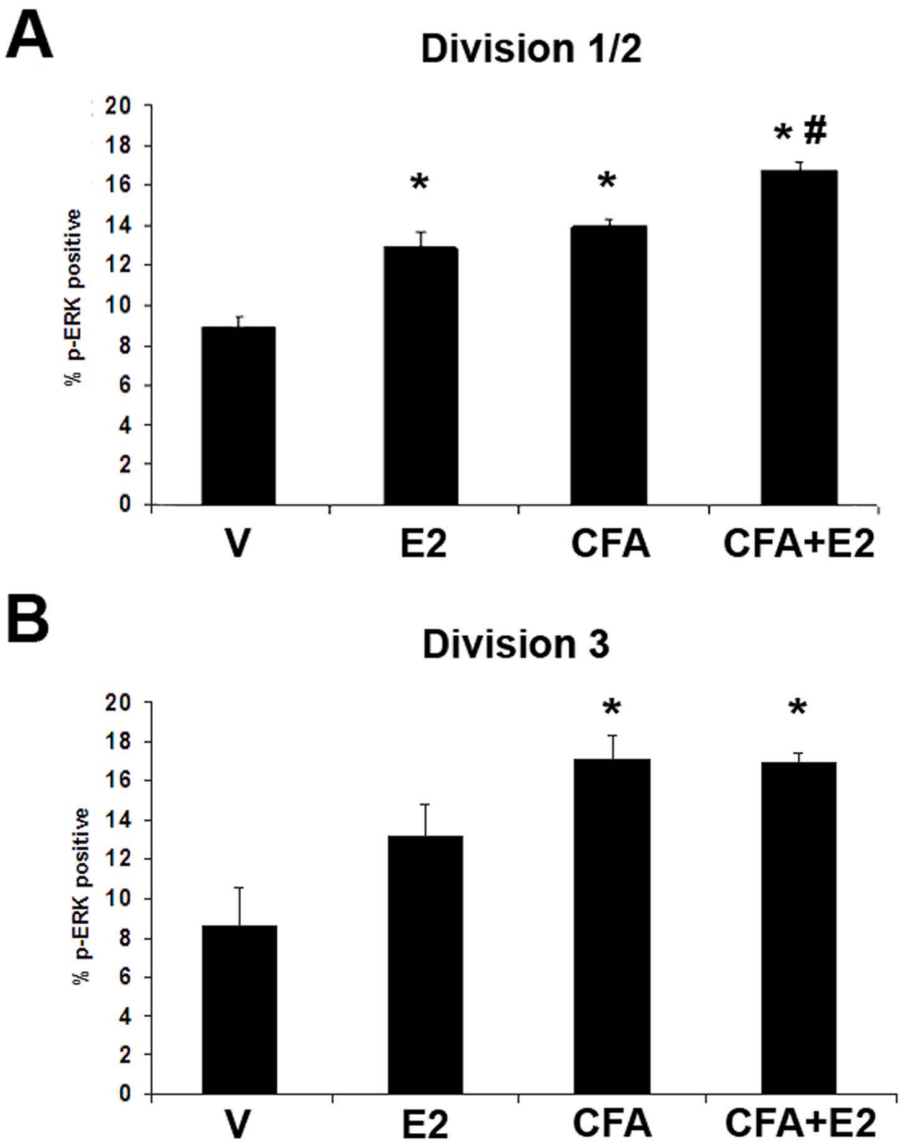

Figure II-6 Estrogen replacement and inflammation increase ERK activation in trigeminal ganglion. Quantification of activated ERK immunoreactivity in division 1/2 (A) and division 3 (B) of trigeminal ganglion sections from ovariectomized female rats treated with CFA, E2, CFA+E2, or vehicle. Mean percentage of pERK immunoreactive neuronal profiles relative to total neuronal profiles per section are shown for each group. For each animal, the left masseter of was injected intra-muscularly with 50 μl CFA (1:1 in saline) or vehicle and estradiol valerate (10μg/kg in sesame oil) or vehicle was administered subcutaneously. Data are shown as mean +/- SEM. N=6 per group; * $p<0.05$ compared to vehicle and # $p<0.05$ compared to CFA, one-way ANOVA.

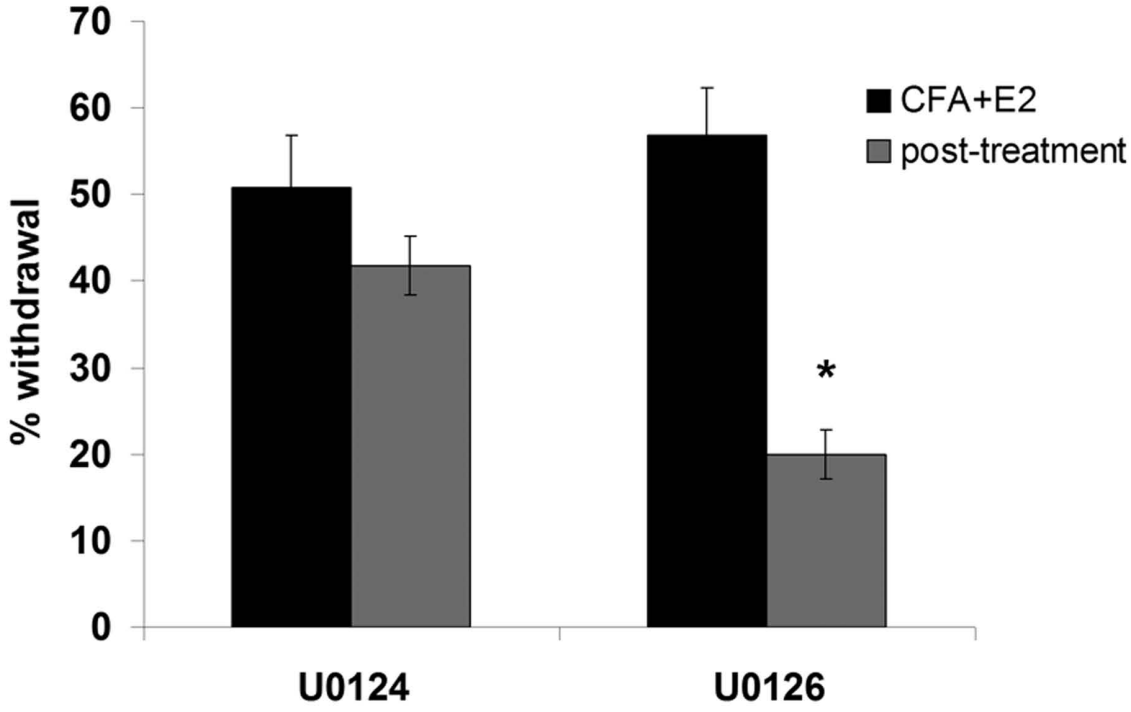

Figure II-7 MEK1/2 inhibitor U0126 delivered to the trigeminal ganglion reduces sensitization following inflammation and estrogen. For each animal, the left masseter of was injected intra-muscularly with 50 μl CFA (1:1 in saline) and estradiol valerate (10 μg/kg in sesame oil) was administered subcutaneously. Withdrawal response to stimulation of the whisker pad with a von Frey filament (0.16g) was assessed 24 hours following CFA+E2 treatment. U0126 or U0124 control was delivered to the trigeminal ganglion by stereotactic injection. Withdrawal response was assessed 24 hours following U0126 or U0124 treatment. Data are presented as mean +/- SEM. N=8 per group; * $p<0.05$ compared to U0124 treatment, one-way ANOVA.

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III. Role of the estrogen receptors GPR30 and ERα in peripheral sensitization: relevance to trigeminal pain disorders in women

Abstract

Estrogen increases facial allodynia through its actions on activation of the MAP kinase ERK in trigeminal ganglion neurons. This goal of study was to determine which estrogen receptor is required for behavioral sensitization. Immunohistochemical studies demonstrated the presence of estrogen receptor alpha (ERα) in nuclei of larger neurons and cytoplasm of smaller neurons, and the novel estrogen receptor G-protein coupled receptor 30 (GPR30) in small diameter neurons that also contained peripherin, a marker of unmyelinated C-fibers. Selective agonists for ERα (PPT) and GPR30 (G-1), but not ERβ (DPN), activated ERK in trigeminal ganglion neurons *in vitro*. Both G-1 and PPT treatment increased allodynia after CFA injections in to the masseter of ovariectomized Sprague-Dawley rats. Treatment with estrogen increased expression of ERα but not GPR30, while masseter inflammation increased GRP30 but not ERα. Differential modulation of these ERK-coupled receptors by estrogen and inflammation may play a role in increased trigeminal pain during periods of falling estrogen.

Introduction

Trigeminal pain disorders such as migraine and temporomandibular disorder (TMD) are among the most common neurological conditions (Rasmussen et al., 1991; LeResche, 1997; Lipton et al., 2001a) and are two to three times more prevalent in women than in men (LeResche, 1997; Lipton et al., 2001b) The sex disparity in the prevalence of trigeminal pain has been attributed to fluctuations in estrogen (Somerville, 1972), and trigeminal pain is concentrated during the reproductive years, often beginning at menarche and declining after menopause (LeResche et al., 2005). Trigeminal pain varies across the menstrual cycle, increasing during the perimenstrual period (MacGregor and Hackshaw, 2004), and in some cases at mid-cycle (LeResche et al., 2003). Although clinical data suggest that estrogen increases susceptibility to migraine, the fall in serum estrogen levels at the time of menstruation may precipitate an attack, suggesting a complex relationship between estrogen levels and the severity of trigeminal pain.

Exogenous estrogens can also affect nociception in the trigeminal system. For example, estrogen replacement therapy decreases thermal pain thresholds (Fillingim and Edwards, 2001) and may trigger migraine with aura (MacGregor, 1999). Oral contraceptive use increases the risk of TMD pain (LeResche et al., 1997). These effects may result from estrogen acting directly on the trigeminal system, which is rich in estrogen receptors (Bereiter et al., 2005).

Estrogen may affect a cell through classical nuclear pathways or by signal transduction cascades initiated at cell surface membrane receptors. These estrogen receptors may include the well characterized estrogen receptor α (ERα), estrogen

receptor β (ERβ), or the more recently described GPR30, a G-protein coupled receptor that has been identified as an estrogen receptor (Revankar et al., 2005; Thomas et al., 2005; Funakoshi et al., 2006). In response to estrogen, GPR30 activates downstream second messenger pathways, including the MAPK extracellular-signal regulated kinase (ERK) (Filardo et al., 2000). ERK activation is a specific marker of nociceptor activation (Ji et al., 1999) that is required for thermal and mechanical hyperalgesia in several models of neuropathic and inflammatory pain (Obata et al., 2004b, a). Estrogen increases facial allodynia through activation of ERK in trigeminal ganglion neurons (Liverman et al., 2007), but the specific receptor mediating these changes has not been determined.

In order to better characterize the effects of estrogen on trigeminal nociception, we analyzed the distribution of GPR30 and the classical estrogen receptor ERα in the trigeminal ganglion and investigated the effects of selective agonists for ERα or GPR30 on ERK activation and facial allodynia. In order to determine how estrogen receptors may be regulated by estrogen and inflammation, we examined changes in protein expression of ERα and GPR30 in the presence and absence of estrogen and peripheral inflammation. Our results support the hypothesis that GPR30 is a mediator of estrogen-related trigeminal nociception and indicate that either GPR30 or ERα can mediate trigeminal sensitization. Furthermore, differential regulation of these receptors by estrogen and inflammation may contribute to increased migraine and TMD attacks during the falling estrogen phase of the menstrual cycle.

Methods:

Animals: Female Sprague-Dawley rats aged 60-75 days (Harlan, Indianapolis, IN) were used for all studies. Rats were maintained on a daily 12h light, 12 h dark schedule with *ad libitum* access to water and food (Harlan Teklad 8604). All studies were conducted in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Ovariectomy: Rats were rapidly anesthetized with 4% isoflurane in compressed oxygen, and anesthesia was maintained on 1% isoflurane. Dorsal bilateral incisions were made midway between the lower ribs and the iliac crest, and both ovaries were isolated and removed. Both incisions were closed with suture clips. Animals then received Buprenex (0.1mg/kg) and were allowed to fully regain consciousness before returning to the animal facility.

Western blot: Trigeminal ganglia were harvested and homogenized in cold lysis buffer (20 mM Hepes buffer, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 mg/ml pepstatin A, 10 mg/ml leupeptin and 10 mg/ml aprotinin) using a Dounce homogenizer. The homogenate was centrifuged at 12,000 g (10 min, 4 ° C) and the supernatant collected. Total protein concentrations were measured with a bicinchoninic acid (BCA) assay kit using bovine serum albumin as a standard (Pierce Biotechnology, Inc., Rockford, IL). Proteins were separated using SDS-PAGE on 12%TRIS-HCl gels (BioRad, Hercules, CA) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes

were subsequently probed with a rabbit anti-GPR30 antibody raised against the C-terminal domain of human GPR30 (1:500, LifeSpan) or rabbit anti-estrogen receptor α (MC-20, 1:300, Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA) was used to control for sample loading. Membranes were rinsed in TBS-Tween-20 and IRDye 800 conjugated anti-goat (Rockland Immunochemicals, Gilbertsville, PA) and Alexa 680 conjugated anti-rabbit (Invitrogen, Eugene, OR) secondary antibodies were applied 1:10,000 in blocking buffer for 1 hour at room temperature. Membranes were rinsed in TBST followed by TBS and visualized using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Band intensities were determined using Odyssey software version 1.0 (LI-COR, Lincoln, NE) and reported relative to GAPDH.

Immunohistochemistry: Two weeks following ovariectomy, rats were deeply anesthetized with sodium pentobarbital (50mg/kg i.p.) and perfused transcardially with 0.1M PBS followed by 4% buffered paraformaldehyde. Trigeminal ganglia were removed, post-fixed overnight in 4% buffered paraformaldehyde and cryoprotected in 30% sucrose in 0.1 M phosphate buffer pH 7.2. Ganglia were frozen in Tissue Freezing Medium (Triangle Biomedical Science, Durham, NC) at -80° C. Frozen sections (20μm) through the horizontal plane of the trigeminal ganglion were prepared using a cryostat (Carl Zeiss, Inc., Oberkochen, Germany). Horizontal sectioning of the ganglion allows visualization of the three anatomic divisions in a single section. The sections were permeabilized and blocked for 1 hour in a solution of 0.2% Triton-X100 and 2% normal goat serum in 0.1M PBS. GPR30 was labeled by incubating overnight at 4°C with rabbit anti-GPR30 (1:300, LifeSpan Biosciences)

followed by 1 hour in AlexaFluor 568 goat anti-rabbit in PBS (1:500, Molecular Probes, Carlsbad, CA). For double-labeling experiments, slides were incubated overnight in rabbit anti-GPR30 antibody and either chicken anti-neurofilament H antibody (NFH, 1:300, Chemicon, Temecula, CA), chicken anti-peripherin antibody (1:300, Chemicon), or mouse anti-ERα antibody (Abcam, 1:100). AlexaFluor 568 conjugated goat anti-rabbit was used as a secondary antibody with either AlexaFluor 488 goat anti-mouse antibody or AlexaFluor 488 goat anti-chicken antibody (1:500, Molecular Probes). Slides were coverslipped using ProLong Gold anti-fade reagent (Molecular Probes).

Microscopy and analysis: Fluorescent digital images were obtained using a Nikon Eclipse 90i upright microscope and a Nikon C1 confocal imaging system using a 20x objective and a frame size of 1024 × 1024 pixels. Sections processed omitting the primary antibody were used to control for non-specific fluorescence. Detector gain and laser intensity were initially set using control slides, and all images were subsequently collected under the same photomultiplier detector conditions and pinhole diameter. In double-label experiments, single-antibody labeled sections were used to control for channel bleed-through, and multi-channel images were acquired using sequential excitation. For cell counting, individual images were montaged into a single image using Adobe Photoshop CS2 to prevent double-counting at the image edges. The peak intensity of cells processed in the absence of primary antibody determined the threshold for counting a cell as GPR30 positive. At least 1000 cells were counted from each of 4 animals for each condition. At least 100 neurons were measured per trigeminal ganglion section.

Single and double-labeled neurons were counted from digital images to obtain a percent score for colocalization of GPR30 with each phenotype marker. The percentage of neurons labeled with both markers was determined by counting the total number of neurons containing GPR30 immunoreactivity and identifying the number of cells labeled with the second neuronal marker. The same procedure was repeated to count the Alexafluor 488 labeled cells. We determined the percentage of peripherin and NFH immunoreactive neurons that also contain GPR30 by dividing the number of double-labeled neurons by the total number of neurons that were immunoreactive for each marker. The experiment was repeated using two sets of independent samples.

Cell diameters were measured using the ruler function of Adobe Photoshop CS2. The length of the short and long axes of each neuron in every selected file was measured. The diameter of each cell was calculated as the average of length and width. Data are reported as mean ± standard error, with a *P* value less than 0.05 using a two-tailed t-test considered significant.

Tissue Culture. For each experiment, trigeminal ganglia from 4-5 cycling female rats were dissected from the base of the skull, rinsed in Hank's balanced salt solution, minced and suspended in digestion medium containing Leibovitz L15 medium with 1 mg/mL bovine serum albumin (BSA), 250 U/mL of CLSPA collagenase, 1 U/mL of ESL elastase, 5 U/mL of PAPL papain (enzymes from Worthington Biochemical Corp., Lakewood, NJ, USA). An equal volume of 30% Stractan (Larex, White Bear Lake, MN, USA) in Leibovitz's L15 was added to the digest. After mixing by inversion, samples were centrifuged in a swinging bucket rotor at 4°C or 10 minutes

at 500*g* to enrich the culture for neuronal cells. The pellet of trigeminal cells was suspended in a small volume of neurobasal A medium (Gibco/Invitrogen, Carlsbad, CA). The cells were plated onto poly-d-lysine coated glass coverslips (Becton Dickinson) and maintained in phenol red-free neurobasal A medium containing 10% fetal bovine serum, 50 ng/mL nerve growth factor, B27 supplement, and 20 *μ*M cytosine arabinoside. Serum and NGF were removed 24 hours prior to pharmacologic treatment to reduce baseline ERK phosphorylation. Experiments were repeated using at least 5 independent tissue cultures.

In vitro pharmacologic treatments: We used synthetic 17*β*-estradiol (Sigma E-2758, St. Louis, MO, USA), the most potent and receptor-specific form of estrogen, to study the effects of estrogen. After waiting for 3 days to allow any endogenous estrogen to be metabolized, 17*β*-estradiol was added to the cultures at a final concentration of 10^{-8} M. The 17*β*-estradiol was initially dissolved in ethanol at a concentration of 10^{-1} M, then further dilutions were performed in phosphate-buffered saline (PBS) and 0.1% BSA to achieve a final concentration of 10^{-8} M, corresponding to a physiological range of plasma estrogen. Cells in the control wells received the diluent only. After 72 hours in culture, cells were treated with 10 nM β-estradiol (Sigma, St. Louis, MO), 0.1 M PBS, or selective agonists for ERα— 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, 10 nM Sigma, St. Louis, MO) (Stauffer et al., 2000), GPR30—G-1 (1 μM, Chemical Diversity, San Diego, CA (Bologa et al., 2006), or ERβ—diarylpropionitrile DPN (100 nM, Sigma).

Immunocytochemistry: Following pharmacologic treatments, cells were fixed for 10 min in 4% paraformaldehyde at room temperature, rinsed, then permeabilized for 15

minutes in 0.2% Triton X-100 and blocked for 1 h in 1X PBS containing 5% normal goat serum. Coverslips were incubated in rabbit anti-p-ERK (1:200, Cell Signaling Technology) and mouse anti-NeuN (1:1000, Chemicon, Temecula, CA) in 1X PBS containing 3% NGS overnight at 4°C followed by washing and secondary antibody staining with goat anti-rabbit Alexa Fluor 568 and goat anti-mouse Alexa Fluor 488 (1:200, Molecular Probes) for 30 min at 25°C. Images were acquired at 40x magnification using a Nikon confocal microscope. A minimum of 100 cells from three independent cultures were analyzed for each experimental group. Cells immunoreactive for NeuN were scored as p-ERK positive or negative by an observer who was unaware of the treatment group.

Orofacial sensitivity assay: Mechanical sensitivity of the orofacial region was assessed using the behavioral assay described previously (Liverman et al., 2007). Briefly, rats were conditioned to traverse a clear plastic tunnel attached to a behavioral arena in order to gain access to a bottle containing 0.1M sucrose. During each session, the rats consume approximately 2 mL sucrose, less than 10% of their total daily water intake. This amount of sucrose consumption is less than 1% of the amount required to produce antinociceptive effects in adult rats, approximately 1M sucrose *ad libitum* over three to four weeks (Kanarek et al., 2001). Importantly, the acute effects of sucrose on analgesia, present in neonates, disappear by P21 (Anseloni et al., 2002). The rats were not restrained in any way, and the tunnel is large enough for the rat to turn around to reenter the arena at any time. Rats were coded and experimenters blinded to experimental groups. Withdrawal behaviors were assessed in response to stimulation of the whisker pad or skin overlying the masseter using 0.16 g and 4 g monofilaments, respectively. Preliminary studies

established that these filaments produced withdrawal responses at or below threshold in control rats. Mechanical withdrawal responses were measured 24, 48, and 72 hours following CFA in combination with selective agonist treatments.

CFA and selective agonist treatment: Two weeks following ovariectomy, rats were given a single 50 µl injection of Complete Freund's Adjuvant (CFA; Mycobacterium tuberculosis H37 Ra 1 mg/ml; Sigma, St. Louis, MO) emulsion, 1:1 in 0.1M phosphate buffer into the left masseter muscle under isoflurane anesthesia. This dose produces molecular and behavioral effects that last for 3 days (Ro, 2005; Lee and Ro, 2007) when injected into the masseter. Control rats were injected with an equal volume of isotonic saline. Estradiol valerate (E2, Delestrogen, Monarch Pharmaceuticals, Bristol, TN, 10 μg/kg in 100μl sesame oil vehicle) was injected subcutaneously using a 22 gauge needle during the same surgery as CFA injection into the masseter. Estradiol valerate is an estradiol conjugate that provides prolonged stable serum estrogen levels (Oriowo et al., 1980). All control rats received sesame oil vehicle. Post-mortem examination of CFA-treated rats and hematoxylin and eosin staining of sectioned facial structures revealed granulomatous inflammation confined to the masseter muscle. We did not observe inflammatory pathology upon examination of cranial sites outside the masseter, including the whisker pad. This is supported by other studies using CFA injection of TMJ and masseter that demonstrate inflammation confined to the injection site (Ogawa et al., 2003; Takeda et al., 2005; Ambalavanar et al., 2006b). Behavioral experiments were conducted with three groups of 10-12 rats. Each rat was given a CFA injection into the masseter muscle and either the ERα–selective agonist PPT, (Sigma, St. Louis, MO, 1mg/kg dissolved 1 μg/ul in sesame oil), the GPR30-selective agonist G-1

(Chemical Diversity, San Diego, CA, 1mg/kg dissolved 1 μg/ul in sesame oil), or sesame oil vehicle by subcutaneous injection between the scapulae using a 22 gauge needle. G-1 is a recently characterized selective agonist for GPR30 (Bologa et al., 2006). PPT is a potent ERα agonist that binds ERα with a 410-fold higher binding affinity than ERβ (Stauffer et al., 2000), and DPN is a selective agonist for ERβ that binds ERβ with a 70 fold higher affinity than ERα (Meyers et al., 2001). The PPT dose was selected according to Harris, 2002. G-1 has been shown to induce sensitization at a range of doses spanning several orders of magnitude (Kuhn et al., 2008).

Statistical analysis

Data were analyzed using Graph Pad Prism version 4.0 software (Graph Pad, San Diego, CA) Behavioral data and immunocytochemistry were analyzed using one-way ANOVA with a Bonferroni post-hoc test. Western blots and cell size measurements were compared using two-tailed unpaired t-tests. Data are presented as mean +/- SEM, with a P value less than 0.05 considered significant.

Results

GPR30 expresssion in rat trigeminal ganglion

In order to determine whether GPR30 protein is expressed in the trigeminal ganglion, we performed Western blots on homogenates of trigeminal ganglia from ovariectomized female Sprague-Dawley rats using a GPR30-specific antibody. Membranes showed a single band of an appropriate molecular weight (Figure III-1) that was eliminated by preincubation of the antibody with the immunizing peptide, indicating that the antibody is specific for GPR30 (data not shown).

GPR30 expression in trigeminal neuron subpopulations

To determine the trigeminal ganglion cell types that express GPR30, we performed immunohistochemistry on frozen sections using the same GPR30 antibody used in Western blots. GPR30 immunoreactivity was localized to neurons (Figure III-2). We observed immunofluorescence throughout the neuronal cytoplasm of positively labeled cells. Immunofluorescence was also specific for GPR30, as preincubation of the antibody with the immunogen peptide eliminated staining (data not shown).

To characterize the expression of GPR30 within trigeminal ganglion sub-populations, we used double-label immunohistochemistry to determine the colocalization of GPR30 with peripherin, a marker of neurons with unmyelinated axons and neurofilament H (neurofilament 200), a marker of neurons with myelinated axons

(Figure III-2). The localization of GPR30 expression was primarily restricted to neurons with unmyelinated axons. Seventy-six percent of GPR30-positive neurons were peripherin positive, and 73% of peripherin-positive neurons were immunoreactive for GPR30. Thirty four percent of GPR30 positive neurons were NFH positive, and GPR30 was present in 32% of NFH positive neurons.

In order to investigate the neuronal phenotypes that express GPR30 in the trigeminal ganglion, we measured the diameters of cells that were immunoreactive and non-immunoreactive for GPR30. GPR30 immunoreactivity was skewed toward small diameter neurons (Figure III-3), although GPR30 was expressed across a broad range of cell sizes. The average diameter of GPR30-positive cells (23.9 ± 0.8 μm) was significantly smaller ($p<0.01$) than the average diameter of GPR30-negative cells (28.9 ± 1.04 μm).

ERα immunohistochemistry

Because ERα is a putative mediator of estrogen-modified nociception, we used immunohistochemistry to label ERα in the trigeminal ganglion. We observed prominent ERα immunoreactivity throughout the trigeminal ganglion that was localized to neurons. ERα was present in both nuclear and cytoplasmic compartments (Figure III-4A). By visual inspection, cytoplasmic ERα immunoreactivity was present prinicipally in small neurons, while nuclear ERα was located in larger cells. In order to quantify the distribution of immunoreactivity, we

measured the diameters of ER immunoreactive cells for both the cytoplasmic and nuclear ERα populations. Figure III-4B shows the diameters of neurons immunoreactive for cytoplasmic and nuclear ERα. Cells expressing cytoplasmic ERα had a mean diameter of 19.1 +/- 0.3 μm, which was significantly smaller ($p<0.05$) than the diameter of cells expressing nuclear ERα, with a mean diameter of 31.2 +/- 0.4 μm.

Colocalization of GPR30 and ERα

We used double-label immunohistochemistry to determine the co-localization of GPR30 and ERα. ERα and GPR30 were present in distinct but partially overlapping populations. We observed neurons that expressed both GPR30 and ERα, and neurons that expressed only ERα or GPR30 (Figure III-5). Ten percent of trigeminal ganglion neurons expressed both GPR30 and ERα, 22% expressed ERα, and 35% expressed GPR30.

ERK activation by selective agonists for ER and GPR30

Estrogen has been demonstrated to activate ERK in primary cultures of dorsal root ganglion and trigeminal ganglion neurons (Purves-Tyson and Keast, 2004; Puri et al., 2006) and in the rat trigeminal ganglion *in vivo* (Liverman et al., 2007). In order to determine which estrogen receptor mediates ERK activation, we assessed ERK activation in primary trigeminal ganglion cultures following application of agonists selective for GPR30, ERα, or ERβ. G-1 or PPT increased the percentage of p-ERK

positive neurons compared to vehicle (Figure III-6). Activated ERK immunoreactivity was present in 67% of PPT-treated neurons and 64% of G-1 treated neurons compared to 18% with vehicle treatment. DPN treatment did not significantly increase ERK activation.

Selective agonists for GPR30 and ERα increase orofacial sensitivity

We previously demonstrated that estrogen increases facial allodynia through activation of ERK (Liverman et al., 2007). To better characterize which estrogen receptor mediates increased trigeminal sensitization, we examined the effects of PPT and G-1 on facial mechanical nociception in the presence of orofacial inflammation using a rat behavioral model. We used Complete Freund's Adjuvant (CFA), an established model of inflammatory pain (Ji et al., 2002; Obata et al., 2004a; Duric and McCarson, 2007) that can induce widespread mechanical hyperalgesia (Ambalavanar et al., 2006a), to induce inflammation of the masseter.

In order to assess facial mechanical sensitivity, we developed a behavioral model based on operant conditioning that allows monofilament testing of the orofacial region. We conducted a preliminary study to determine the monofilament force resulting in a 50% withdrawal threshold in ovariectomized rats. A sub-threshold filament was subsequently used for the testing of whisker pad in order to measure allodynia. In order to assess secondary allodynia, mechanical sensitivity of the whisker pad was measured using a 0.16g monofilament 24, 48, and 72 hours following CFA injection and selective agonist treatment. Injection of either PPT or G-

1 enhanced withdrawal responses in the setting of peripheral inflammation at 24 hours compared to vehicle-treated controls (Figure III-7).

Estrogen effects on expression of GPR30 and ERα

In order to determine whether estrogen treatment changes the protein content of estrogen receptors in the trigeminal ganglion, we used Western blot to compare ERα and GPR30 protein levels in trigeminal ganglion samples from OVX and OVX+E2 rats. We observed a significant increase in ERα in ganglia from estrogen-treated animals compared to vehicle-treated controls (Figure III-8). Two size variants of ERα were present in the trigeminal ganglion, at 66 kD and 90 kD. Both variants were upregulated by estrogen, 1.81-fold and 1.76-fold respectively. GPR30 protein content was not significantly altered in the trigeminal ganglion by estrogen replacement.

Inflammation effects on expression of GPR30 and ERα

In order to determine whether peripheral inflammation of the trigeminal nerve alters expression of ERα and GPR30 in the trigeminal ganglion, we compared ERα and GPR30 protein levels using Western blots of trigeminal ganglion samples taken from groups of ovariectomized rats with inflamed or normal masseter muscles. Inflammation of the masseter increased GPR30 expression 2.25 fold in the trigeminal ganglion compared to vehicle-treated

controls, whereas it did not significantly change ERα expression in the trigeminal ganglion (Figure III-9).

Discussion:

Although it is well established that estrogen modifies orofacial pain, the receptors mediating these changes have remained unclear. Results of the current study support roles for both the novel estrogen receptor GPR30 and estrogen receptor α in estrogen-modified nociception. Furthermore, our data show that these receptors are differentially regulated by estrogen and inflammation, which may have important implications for the modulation of trigeminal pain during the menstrual cycle.

GPR30 is present in the trigeminal ganglion

Our results show that GPR30 is present in the trigeminal ganglion of female rodents. GPR30 expression was localized to small, unmyelinated, peripherin-positive neurons, suggesting that GPR30 is localized to nociceptors in the trigeminal ganglion. Identification of a novel estrogen receptor in the sensory pathway is potentially significant to estrogen-modified trigeminal sensitization. Female animals are more sensitive to trigeminal nociception than males (Bereiter, 2001; Cairns et al., 2002) especially during the high estrogen phase of the estrous cycle (Okamoto et al., 2003; Martin et al., 2007). Furthermore, estrogen replacement increases excitability of trigeminal afferents and enhances inflammatory sensitization of the trigeminal

system (Bereiter and Barker, 1980; Flake et al., 2005; Liverman et al., 2007). The presence of GPR30 in cells likely to be nociceptors suggests that GPR30 is a possible mediator of estrogen-modulated pain sensitization.

ERα is present in both cytoplasmic and nuclear compartments

ERα immunoreactivity was present in both cytoplasmic and nuclear compartments of trigeminal ganglion neurons. Previous studies of ERα in the rat trigeminal ganglion have restricted analyses to cells with nuclear labeling, discounting the potential importance of cytoplasmic estrogen receptor immunoreactivity (Bereiter et al., 2005; Puri et al., 2006). The results of this study demonstrate a size differential based on subcellular localization, with cytoplasmic ERα predominant in smaller neurons and nuclear ER in larger neurons. These results are intriguing in light of the dual role of ERα in both classical nuclear pathways and 'non-genomic' actions through signal transduction pathways. ERα-dependent activation of signal transduction molecules is thought to originate from receptors sequestered in cytoplasmic signaling complexes (Manavathi and Kumar, 2006). The current results suggest that this mechanism predominates in nociceptive neurons, while, in larger diameter neurons, where ERα immunoreactivity is localized to the nucleus, estrogen may preferentially function through direct genomic mechanisms. Previous studies have shown that estrogen modifies nociception through non-genomic mechanisms by activating ERK in the trigeminal ganglion (Liverman et al., 2007). The present results suggest that cytoplasmic ERα in small diameter neurons contributes to this process.

ERα and GPR30 are expressed in distinct populations

Because the present data show that cytoplasmic ERα and GPR30 are expressed in small-diameter neurons, the co-localization of ERα and GPR30 in the trigeminal ganglion was analyzed. Results show that ERα and GPR30 are present in distinct, but partially overlapping, neuronal populations, and that the majority of small trigeminal neurons express one of these receptors. Expression of either ERα or GPR30 by the majority of nociceptors in the trigeminal ganglion suggests a basis for the high sensitivity of the trigeminal system to estrogen.

ERK is activated through GPR30 and ERα

Results show that selective agonists for either GPR30 or ERα induce rapid ERK activation in trigeminal ganglion neurons, suggesting that ERK activation mediated by estrogen (Puri et al., 2006; Liverman et al., 2007) can occur through either estrogen receptor α or GPR30. Furthermore, these data show that trigeminal GPR30 is functional in activating signal transduction pathways in trigeminal ganglion neurons, which may be relevant to estrogen's modification of nociception.

There is considerable precedent suggesting that ERK can be activated by either ERα or GPR30 in many estrogen sensitive cell types (Vivacqua et al., 2006b; Vivacqua et al., 2006a; Albanito et al., 2007), and signaling through either receptor has been demonstrated in the same cell type (Albanito et al., 2007).

Estrogen receptor-evoked activation of ERK may be significant to the pathogenesis of estrogen-modified sensitization. ERK phosphorylation in sensory neurons is a specific marker of nociceptive stimulation (Ji et al., 1999) and a mediator of mechanical hyperalgesia in pain models of peripheral nerve injury and inflammation (Obata et al., 2003; Obata et al., 2004c). As estrogen increases mechanical allodynia of the orofacial region through activation of ERK (Liverman et al., 2007), ERK phosphorylation resulting from ERα or GPR30 activation may be important to the pathogenesis of trigeminal pain. Activation of ERK in the unmyelinated neurons expressing ERα or GPR30 could lead to increases in neuronal excitability resulting in allodynia.

GPR30 and ERα participate in trigeminal sensitization

The results of the current study demonstrate that selective agonists for either GPR30 or ERα enhanced secondary mechanical allodynia of the orofacial region, suggesting that both GPR30 and ERα are capable of mediating pro-nociceptive responses to estrogen in the trigeminal system. By identifying GPR30 as a putative modulator of sensitization in the trigeminal system, the current results reveal an increased complexity of pro-nociceptive effects of estrogen in the trigeminal system. The presence of GPR30 in the trigeminal ganglion presents a novel pathway through which estrogen may modulate the activity of sensory neurons, and which may be relevant to effects of hormonal fluctuations on migraine and facial pain.

ERα is increased by estrogen while GPR30 is increased by inflammation

Results show that inflammation in the trigeminal distribution upregulates GPR30 protein in the trigeminal ganglion. Although the effect of inflammation on GPR30 expression has not been previously addressed, estrogen and inflammation have been shown to have additive effects on trigeminal excitability and nociceptive responses (Cairns et al., 2002; Flake et al., 2005; Martin et al., 2007). Increased expression of GPR30 in the presence of inflammation may lead to enhanced estrogen signaling. The current data suggest that peripheral inflammation during episodes of trigeminal pain may increase GPR30 and lead to enhanced modulation of nociception by estrogen. Furthermore, since inflammation did not modify expression levels of ERα, peripheral trigeminal inflammation may shift the balance of estrogen signaling in trigeminal neurons toward GPR30.

Data show that the presence of estrogen increases levels of ERα protein in the trigeminal ganglion. The relationship between serum estrogen and ERα receptor expression is complex and appears to be highly dependent on both the estrogen level and tissue studied, but these data are consistent with several previous studies showing that estrogen increases ERα in sensory neurons (Zoubina and Smith, 2003; Bereiter et al., 2005). GPR30 expression, by contrast, was not changed by estrogen replacement. This result is consistent with studies of hamster ovary showing that GPR30 expression was not changed by estrogen replacement (Wang et al., 2007).

The current results suggest two potential mechanisms that may contribute to the paradoxical effect of falling estrogen leading to increased pain. First, ERK activation,

mediated by GPR30 and ERα, may be involved in sensitization during periods of decreased serum estrogen. ERK may be activated intermediate doses of estrogen, but not by high doses of estrogen, suggesting that as estrogen concentrations drop during the menstrual phase, ERK activation may increase (Kuroki et al., 2000). Second, differential regulation of GPR30 and ERα by estrogen and inflammation may contribute to increased pain during periods of falling estrogen. The results of this study show that inflammation in the trigeminal region increases GPR30 expression levels, shifting the balance of estrogen signaling toward GPR30. In addition, falling serum estrogen concentrations would downregulate ERα expression levels, further shifting estrogen signaling toward GPR30. Since the neuronal populations that express ERα and GPR30 are largely distinct, a decrease in serum estrogen concentration in the context of peripheral inflammation could shift estrogen signaling to favor the population that expresses GPR30, which is predominately composed of likely nociceptive neurons. Although the downstream genes differentially induced by activation of GPR30 and ERα are not completely characterized, they may include pro-nociceptive genes.

In conclusion, the results of this study demonstrate that estrogen can modulate trigeminal nociception through both the GPR30 and ERα pathways and provide new insight into the complex relationship between estrogen and trigeminal pain.

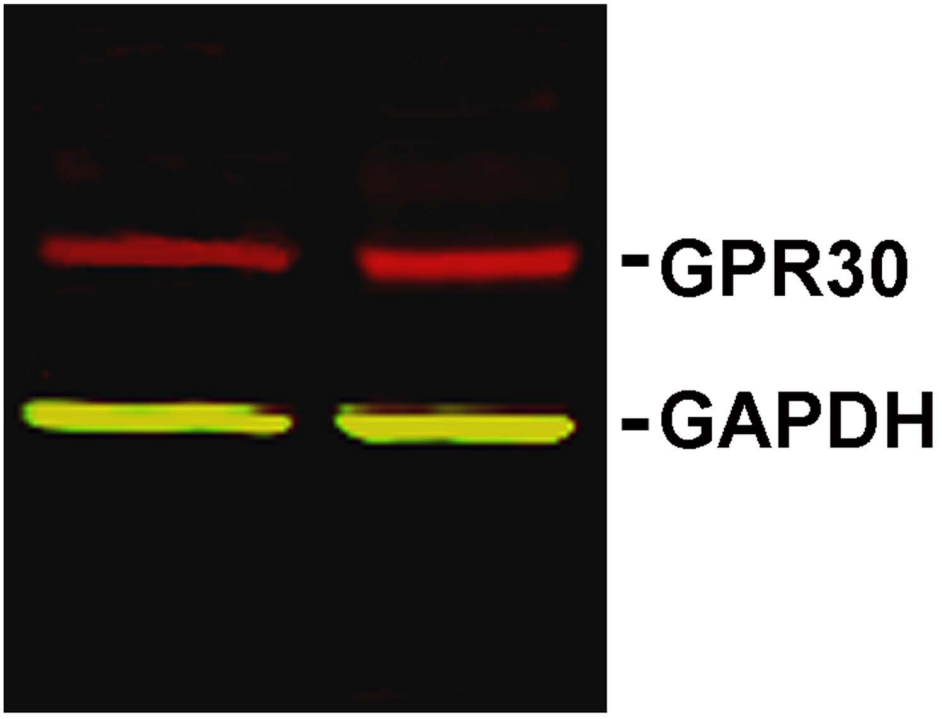

Figure III-1. GPR30 protein is present in rat trigeminal ganglion. Western blot of trigeminal ganglion lysates from ovariectomized female rats labeled with antibodies to GPR30 (red) and the loading control GAPDH (green).

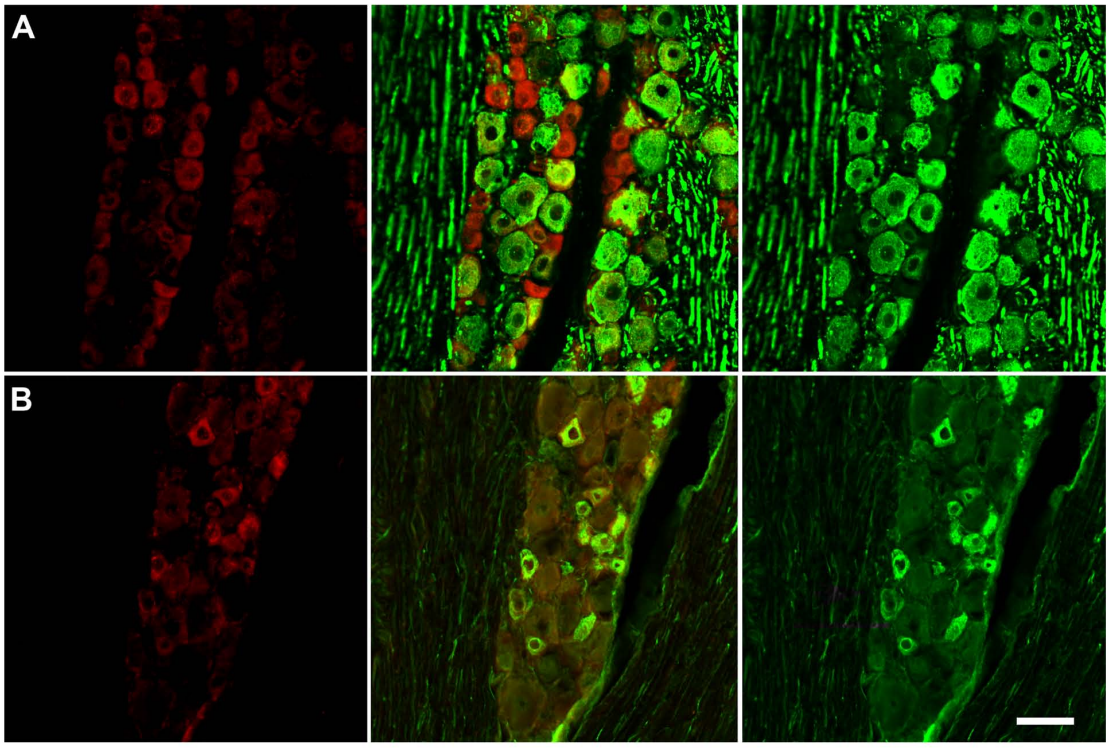

Figure III-2. GPR30 is localized to unmyelinated neurons in the trigeminal ganglion A) Double-label immunohistochemistry for GPR30 and NFH, a marker of myelinated neurons. There is a small degree of overlap between GPR30 and NFH. B) Double-label immunohistochemistry for GPR30 and peripherin. GPR30 and peripherin are highly co-localized. Panels on the left are single-labeled for GPR30. The middle row of panels contains merged images of the left and right columns. The right row of panels are single-labeled neurofilament H (top) or peripherin (bottom). Scale bar (50 μm) applies to all images.

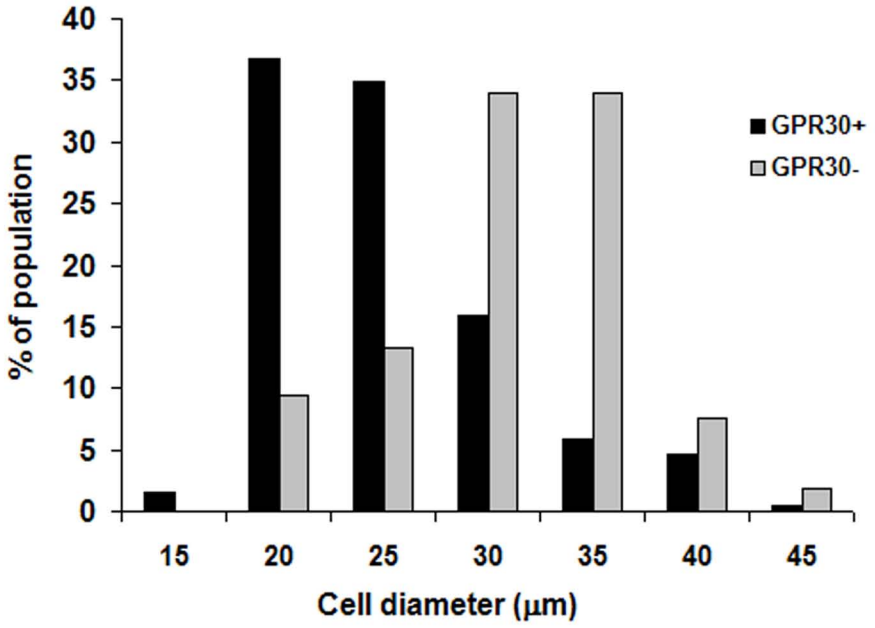

Figure III-3. GPR30 is present in small diameter neurons. Histogram shows cell diameter measurements for trigeminal ganglion neurons that were GPR30 immunoreactive (black bars) and GPR30 non-immunoreactive (gray bars).

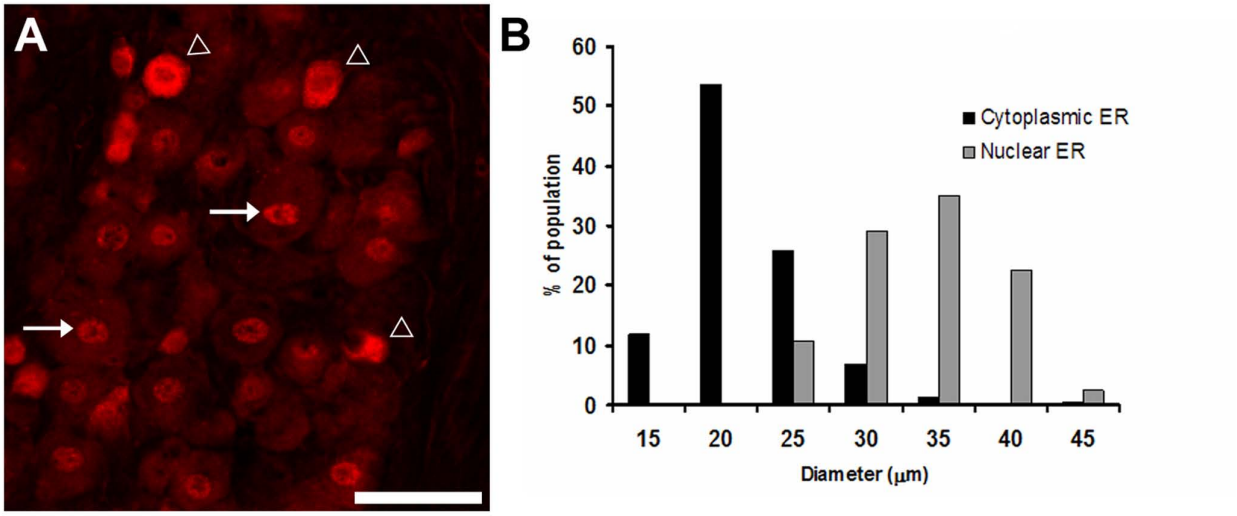

Figure III-4. Cytoplasmic and nuclear ERα immunoreacivity is present in the trigeminal ganglion. A) Immunohistochemistry for ERα in trigeminal ganglion from ovariectomized female rat. ERα staining was present in neurons in both cytoplasmic and nuclear locations B. Histogram showing cell diameter measurements for neurons that were immunoreactive for nuclear and cytoplasmic ERα. Cytoplasmic ERα was present in predominately small neurons and nuclear ERα was present in larger neurons.

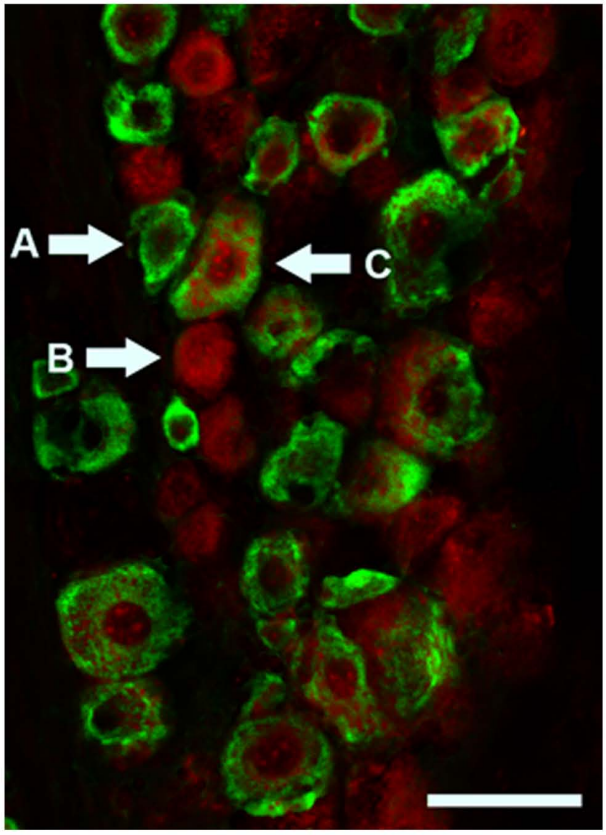

Figure III-5. GPR30 and ERα are present in distinct but overlapping populations in the rat trigeminal ganglion. Immunohistochemistry for GPR30 (red) and ERα (green) shows ERα positive (A), GPR30 positive (B), and ERα/GPR30 double-positive (C) neurons. Scale bar (50 μm)

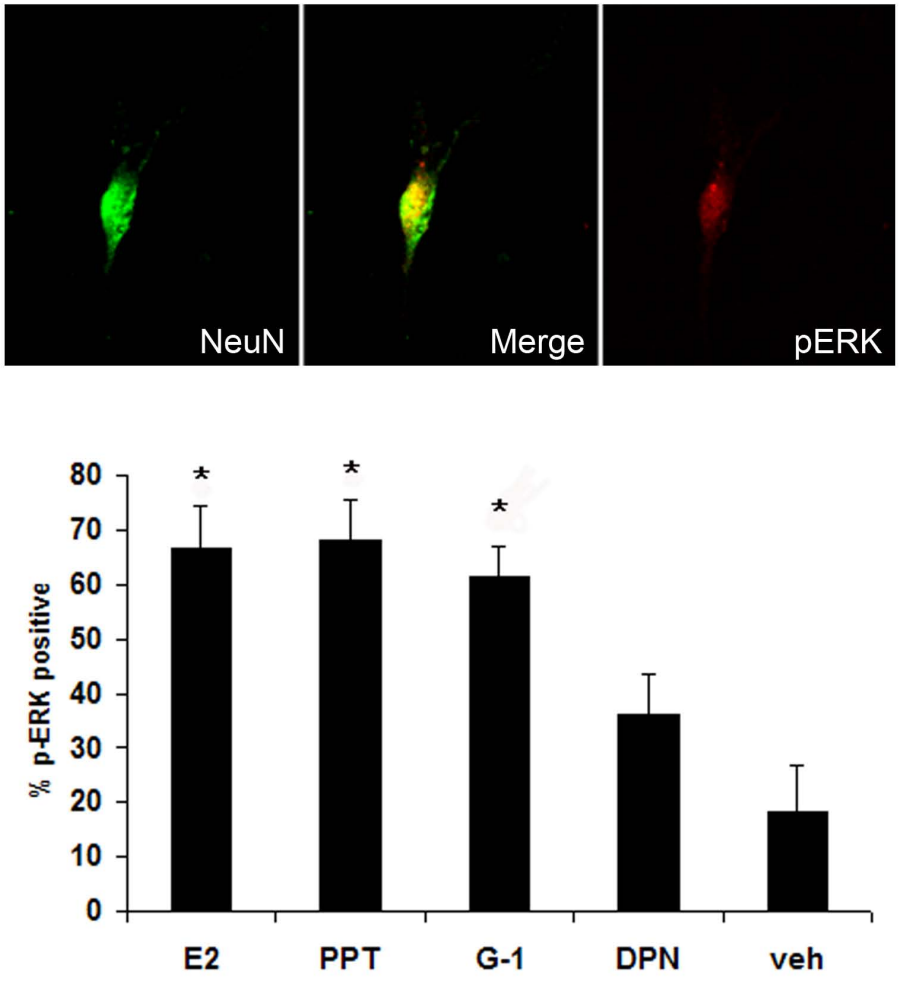

Figure III-6. Selective activation of GPR30 or ERα activates ERK in trigeminal ganglion neurons *in vitro*. Cultured trigeminal ganglion neurons were treated with specific agonists for ERα (PPT, 10 nM), GPR30 (G-1, 1 μM), ERbeta (DPN, 100 nM), 17β–estradiol (E2,10nM), or vehicle. Following agonist treatment, cultures were stained with antibodies raised against the dually-phosphorylated form of ERK and the neuron-specific marker NeuN and the percentage of neurons immunoreactive for activated ERK was assessed. Data are presented as the mean percentage of NeuN positive neurons that were p-ERK positive from three independent experiments. *=p<0.05 compared to vehicle, two-tailed t-test.

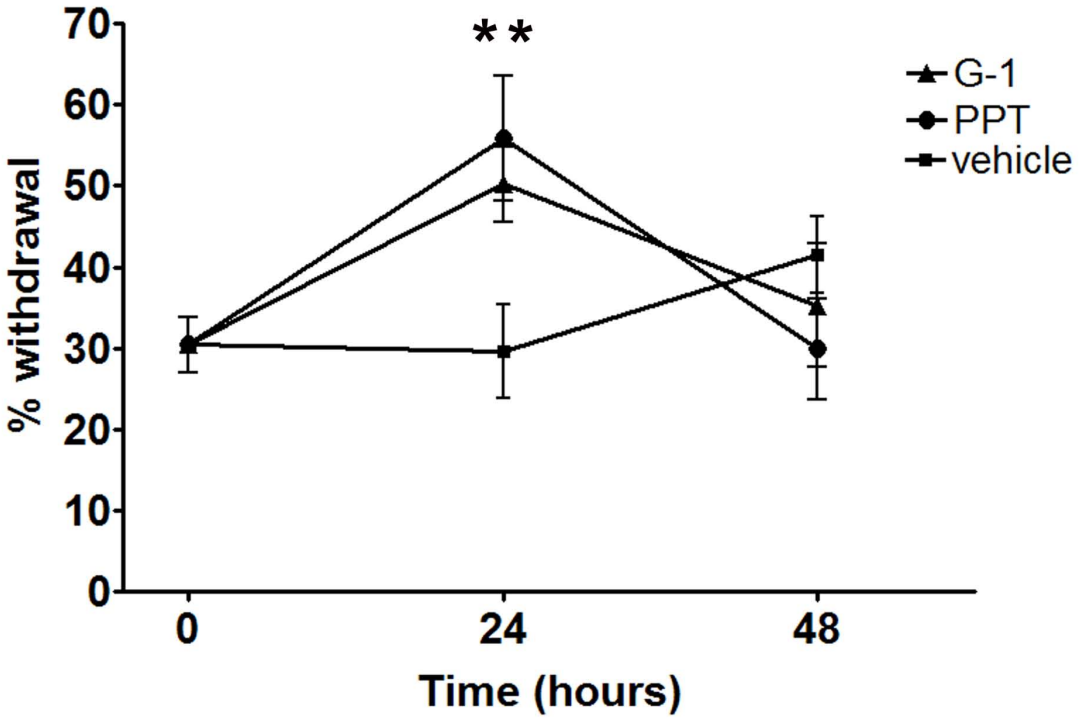

Figure III-7. **Selective activation of GPR30 or ERα enhances orofacial allodynia. Specific agonists for ERα (PPT) and GPR30 (G-1) increased withdrawal response to stimulation of the whisker pad in CFA-treated ovariectomized female rats. For each animal, the left masseter of was injected intra-muscularly with 50 μl CFA (1:1 in saline) and PPT (1 mg/kg), G-1 (1 mg/kg) or vehicle were injected subcutaneously. Withdrawal response to stimulation of the whisker pad with a von Frey filament (0.16g) was assessed at 24 hours after agonist administration. Data are shown as mean+/- SEM. N=8 per group; *=p<0.05 compared to vehicle, two-tailed t-test.**

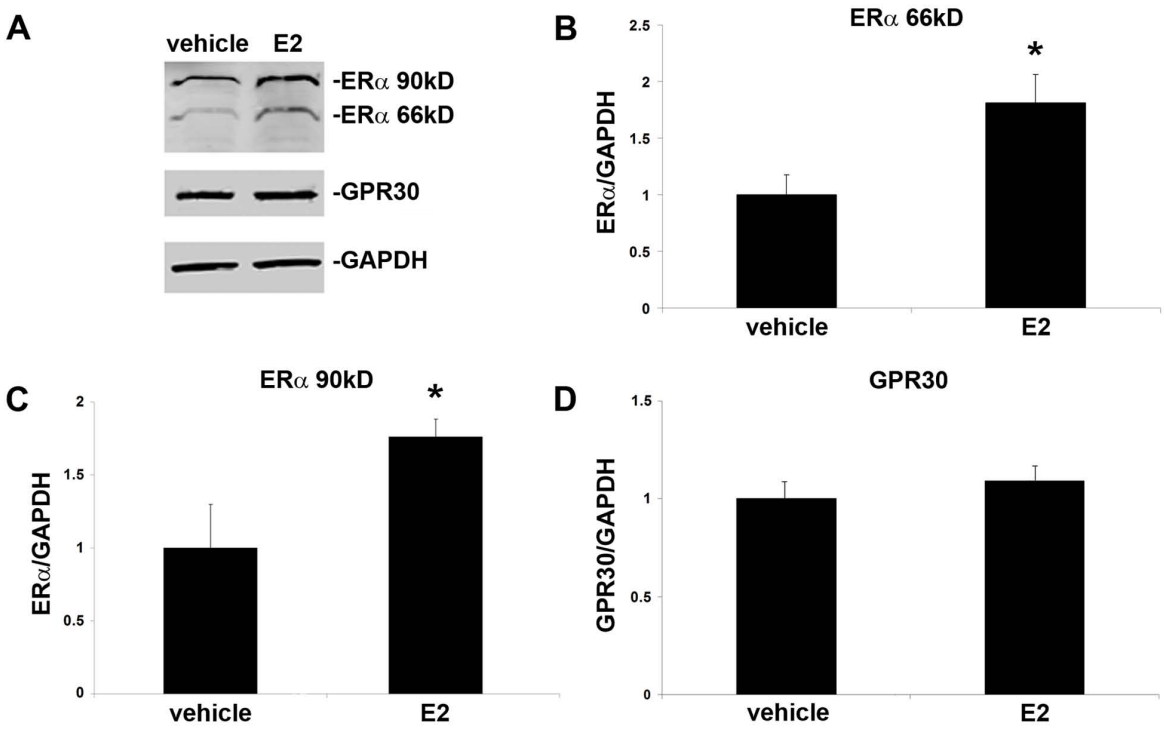

Figure III-8. Estrogen treatment increases expression levels of ERα, but not GPR30, in the trigeminal ganglion of ovariectomized female rats. Estradiol valerate (10μg/kg in sesame oil) or vehicle was administered by subcutaneous injection and trigeminal ganglia collected 24 hours later. A) Representative Western blots of trigeminal ganglion from ovariectomized rats treated with estrogen or vehicle. B) Effects of estrogen on protein level of the 66kD ERα isoform C) Effects of estrogen on the protein level of the 90kD ERα isoform D) Effects of estrogen on GPR30 protein levels. Data are shown as mean integrated intensity relative to GAPDH +/- SEM. (N=6 per group; *=p<0.05 compared to vehicle, one-way ANOVA).

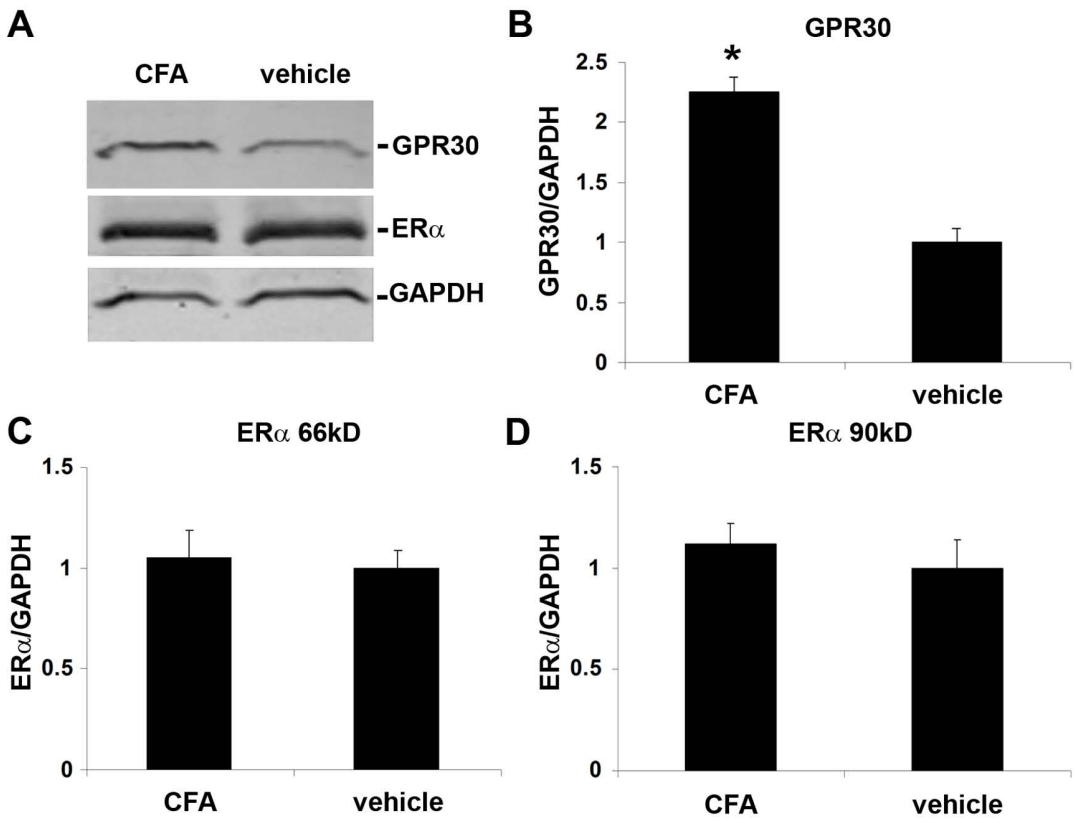

Figure III-9. Peripheral inflammation increases expression of GPR30 but not ERα in trigeminal ganglion from ovariectomized female rats. CFA (1:1 in saline) or vehicle was administered intra-muscularly into the masseter muscle and trigeminal ganglia were collected 24 hours later. A) Representative Western blots of trigeminal ganglion from rats with or without masseter inflammation B) Effects of inflammation of the masseter muscle on GPR30 protein level C) Effects of inflammation of the masseter muscle on ERα protein level. Data are shown as mean integrated intensity relative to GAPDH +/- SEM (N=6 per group; *=$p<0.05$ compared to vehicle, one-way ANOVA).

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IV. Summary and Conclusions

The first aim of this project was to assess the *in vivo* effects of estrogen on inflammatory allodynia. An initial challenge in accomplishing this aim was to establish a reliable method for assessing mechanical allodynia in the facial region. Although there are established methods for evaluating mechanical hyperalgesia in other regions of the body, such as the hindpaw, these techniques prove difficult to apply to the facial region due to the challenge of producing a stationary rat without the use of restraint. The constant exploratory behavior of the rat when exposed to a novel environment such as a testing arena makes it difficult to reproducibly test the same area, to be confident that a constant stimulus is being delivered, and to discern withdrawal responses from spontaneous movements. Although mechanical testing of the orofacial region using graded monofilaments has been described in previous reports (Ren, 1999; Ambalavanar et al., 2006), those methods require the investigator to handle the animal during testing, which alters behavioral responses to stimuli (Chesler et al., 2002).

We overcame those problems by using an operant model in which rats are trained to drink sweetened water from a standard rodent water bottle. During this task, the rat holds its head in a fixed position and focuses its attention on obtaining the reward. This provides increased precision, as the fixed position of the rats head allows the investigator to precisely access specific regions of the face using monofilaments and eliminates variability in the angle of testing. Furthermore, the operant behavior allows the rat to be tested without direct contact by the investigator, which increases the sensitivity of the assay. Models in which rodents are held by the investigator during testing produce withdrawal thresholds that are considerably

higher, >50g for masseter stimulation, than those observed using the present method, approximately 4g. Moreover, although previous methods of trigeminal testing assess whether or not stimulation elicits a response, our method also scores the magnitude of the response. In doing so, this method is sensitive to changes in the degree of sensitization.

We anticipate that the operant method of trigeminal mechanical testing developed in this project will allow trigeminal mechanical nociception to be assessed with increased sensitivity, which will prove valuable to future study of trigeminal pain and for evaluating potential therapeutic interventions.

The behavioral data collected using this method provide evidence that estrogen treatment of ovariectomized rats increases both primary and secondary allodynia of the orofacial region. Specifically, estrogen increases the duration of primary allodynia, and increases both the duration and magnitude of secondary allodynia. More than two decades ago, the studies of Bereiter demonstrated that estrogen increases the size of trigeminal receptive fields (Bereiter and Barker, 1975, 1980). The current findings support a role for estrogen in increasing the size of the sensitized area in the presence of inflammatory nociception. Several reports indicate that estrogen has an additive effect on the increases in trigeminal excitability induced by inflammation (Cairns et al., 2002; Okamoto et al., 2003; Flake et al., 2005; Martin et al., 2007). Our data suggest that these estrogen-induced increases in excitability translate to enhanced nociception. Although estrogen has been shown to induce excitability in the absence of inflammation (Bereiter and Barker, 1980; Flake et al., 2005), there is no increase in trigeminal sensitization with estrogen alone. It is therefore possible that estrogen potentiates the nociceptive effects of inflammation

through increased excitability. In terms of trigeminal pain disorders, the data suggest that circulating estrogen increases the excitability of trigeminal afferents and leads to exaggerated nociceptive input from inflammation. This may result in pain from normally sub-threshold inflammatory processes.

The second goal of this project was to investigate whether ERK activation plays a role in estrogen-modified nociception. Data show that either estrogen or inflammation in the region of the masseter increase ERK activation in the trigeminal ganglion. Moreover, estrogen and inflammation combined produce an additive effect on ERK activation in trigeminal neurons. Building upon previous reports, which showed that estrogen activates ERK in cultured sensory neurons (Purves-Tyson and Keast, 2004; Puri et al., 2006), the results demonstrate that estrogen activates ERK in the trigeminal ganglion *in vivo*. Furthermore, as delivery of an ERK pathway inhibitor to the trigeminal ganglion reduces facial sensitization, the data show that peripheral ERK activation within trigeminal ganglion neurons is an important component of sensitization of the orofacial region. Taken together, the data provide a mechanism linking high estrogen states and increases in excitability of trigeminal ganglion neurons and suggest that estrogen potentiates nociception by increasing excitability through ERK activation.

An important aspect of these results is that antagonizing ERK activation reduces secondary allodynia, which crosses dermatomal borders. This finding challenges the dogma of trigeminal allodynia, which proposes that secondary allodynia is an indication of central sensitization. This mechanism is hypothesized to occur when input from sensitized first-order trigeminal afferents sensitizes second-

order neurons in the trigeminal nucleus caudalis, lowering the activation threshold of convergent first-order neurons. In the trigeminal system, data showing that chemical stimulation of dural afferents enhances the responses of second order nucleus caudalis neurons to stimulation of facial skin have been taken to indicate central sensitization (Burstein et al., 1998). The current observation that secondary allodynia is ameliorated by blocking ERK activation in the trigeminal ganglion after the development of central sensitization calls this interpretation into question, and suggests that processes in the periphery are involved in maintaining secondary allodynia.

The data from experiments in this proposal support a cascade of events in which inflammation of the masseter increases ERK activation outside the third division of the trigeminal ganglion. This is an intriguing finding, as it shows that peripheral inflammation can activate ERK in cell bodies that have no direct chemical or electrical contact with the inflamed tissue. These results suggest that ERK activation can occur through intercellular communication within the ganglion and provide a mechanism by which secondary allodynia can occur independent of central sensitization.

The results in this dissertation are significant to our understanding of the pathogenesis of cutaneous facial allodynia, as they provide evidence that it arises at least in part from processes within the trigeminal ganglion. While there is certainly a contribution of central sensitization in development of allodynia, our data provide evidence that it is not a complete explanation. The presence of allodynia across trigeminal dermatomes alone can no longer be said to demonstrate central sensitization. Conversely, the existence of central sensitization does not preclude

the contribution of intra-ganglionic mechanisms. Intra-ganglionic sensitization may function in conjunction with central sensitization in the generalization of pain during the course of a trigeminal pain episode.

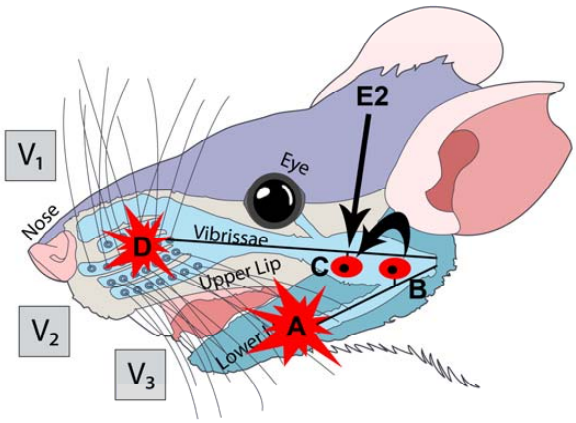

Figure IV-1. Proposed mechanism of secondary allodynia. Inflammation in V3 (A) leads to ERK activation in V3 neurons (B) which communicate (arrow) with V2 neurons (C). Signals from V3 and estrogen (E2) enhance ERK activation in the V2 neuron, lowering the nociceptive threshold of the region innervated by V2 (D).

A possible mechanism for secondary allodynia is shown in the schematic (Figure IV-1). Inflammation in V3 leads to ERK activation in the cell body of a V3 neuron. The signal is transmitted to a V2 neuron, activating ERK, through a process that may involve communication through gap junctions or local release of neurotransmitters. In the presence of estrogen, there is increased ERK activation and increased excitability of the V2 neuron. That process lowers the threshold of V2

nociceptors, and a previously innocuous mechanical stimulus presented to the whisker pad results in nociception.

Taken together, the data suggest that estrogen increases secondary sensitization by broadening the sensitized area and that this occurs through ERK activation in the trigeminal ganglion. These results suggest the possibility that modulation of signal transduction pathways in the periphery following the development of central sensitization may be a useful avenue in the management of trigeminal pain.

The third goal of this project was to determine which estrogen receptors in the trigeminal ganglion function in ERK activation and estrogen-modified nociception. The results demonstrate that GPR30 is present in the trigeminal ganglion and is localized to nociceptors. GPR30 and ERα are present in distinct, but partially overlapping, neuronal populations, and the majority of small trigeminal neurons express one of these receptors. Expression of at least one estrogen receptor by the majority of nociceptors in the trigeminal ganglion suggests a basis for the high sensitivity of the trigeminal system to the effects of estrogen on nociception.

Behavioral data show that activation of either GPR30 or ERα using selective agonists enhances secondary mechanical allodynia of the orofacial region. The results demonstrate that the pro-nociceptive effects of estrogen in the trigeminal system can be mediated by either GPR30 or ERα. Furthermore, we demonstrate that either GPR30 or ERα can activate ERK in cultured trigeminal ganglion neurons. In light of our previous results showing that ERK activation in the trigeminal ganglion

is a mediator of sensitization, the results provide a mechanism by which ERα and GPR30 mediate trigeminal sensitization.

By identifying GPR30 as a putative modulator of sensitization in the trigeminal system, the results provide evidence that pro-nociceptive effects of estrogen in the trigeminal system are more complex than was previously believed. The presence of GPR30 in the trigeminal ganglion presents a novel pathway through which estrogen may modulate the activity of sensory neurons, and which is likely relevant to the effects of hormonal fluctuations on migraine and facial pain.

The data provide evidence that peripheral inflammation increases expression of GPR30 but not ERα in the trigeminal ganglion, while estrogen increases expression of ERα but not GPR30. Differential regulation of GPR30 and ERα may contribute to increased pain during periods of falling estrogen. In the setting of peripheral inflammation, estrogen signaling would shift in the direction of GPR30. Falling serum estrogen concentrations, by decreasing ERα expression, would also shift estrogen signaling toward GPR30. Since the neuronal populations that express ERα and GPR30 are largely distinct, a decrease in serum estrogen concentration during ongoing peripheral inflammation would render the population expressing GPR30, predominately nociceptors, more responsive to estrogen, leading to sensitization.

Taken together the results suggest that both the novel estrogen receptor GPR30 and ERα participate in estrogen-modified nociception through the activation of ERK. Differential regulation of these receptors may have important implications for modulation of trigeminal pain by estrogen.

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