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Contributions of spinal estrogenic neurons to pain and itch

by

May Tran

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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[redacted]

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ABSTRACT

In both acute and chronic pain, females demonstrate greater sensitivity than males. One possible cause for this sex difference is a neural action of estrogen, the primary female sex hormone. To better understand the nociceptive circuits in which estrogen may act, we examined the neuronal expression of aromatase, the enzyme that catalyzes the conversion of testosterone to estrogen, in the spinal and medullary dorsal horns as well as the behavioral outcome of ablating estrogen receptor α (ERα)-expressing neurons in the superficial dorsal horn of the spinal cord. In the first study, we defined the distribution of aromatase-expressing cells in the spinal cord, caudal spinal trigeminal nucleus, and nucleus of the solitary tract in male and female aromatase reporter mice. Immunostaining for markers of neuronal subpopulations established that the aromatase-expressing cells are inhibitory interneurons that are concentrated in laminae I and V. A small percentage of these cells respond to algesic and pruritic agents.

In the second study, we determined that spinal ERα^{+} cells are primarily excitatory interneurons in lamina II that express the nociceptive neuropeptide substance P and receive input from myelinated primary afferents. To assess the function of the ERα-expressing neurons, we ablated these cells by injecting a Cre-dependent caspase virus into the spinal cord of both male and female ERα-Cre mice. In a battery of nociceptive and pruriceptive behavioral testing, we found that ablation reduced formalin-induced nocifensive behaviors and chloroquine-induced pruritofensive behaviors in a sexually dimorphic fashion with no impact on mechanical and thermal thresholds, capsaicin- or histamine-induced responses, or against thermal and mechanical hypersensitivity in models of chronic inflammatory and neuropathic pain. In total,

we have defined two distinct populations of interneurons in the superficial dorsal horn: an inhibitory subset that can synthesize estrogen via aromatase and an excitatory subset that can respond to estrogen via ERα. These studies, although not defining the distinct contribution of estrogen to the modulation of pain and itch messages, provide evidence that these subpopulations are ideally positioned to influence pain and itch processing.

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CHAPTER 1

Introduction

1-1. AN OVERVIEW OF PAIN, ITCH, AND SEX DIFFERENCES

Pain

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (IASP Task Force on Taxonomy, 2012). The pain one feels when touching a hot stove or stepping on a sharp nail is certainly an unpleasant experience, but that very unpleasantness is a warning that the body is in imminent danger and that measures should be taken to avoid further harm. As such, this kind of pain, known as acute pain, is beneficial and contributes to our survival. However, chronic pain, in which pain continues for months or years, in many instances without apparent cause, is maladaptive and has become a serious public health issue given the dearth of safe and effective treatments (Turk, 2002; Goldberg & McGee, 2011; Tompkins et al., 2017). In the United States, approximately 15% of adults—almost 38 million people as of 2017—suffer from moderate-to-severe pain that persists for at least three months (Verhaak et al., 1998; Nahin, 2015). The resulting healthcare expenses, lost productivity, and decreased quality of life thus hurt not only the individual, but also society as a whole, wherein chronic pain's estimated annual cost to the US economy is over 100 billion dollars (Phillips, 2009; Gaskin & Richard, 2012). The demand

for better pain management strategies is immense, but in pursuit of these therapies, we must understand the mechanisms that govern pain function and dysfunction.

Basic anatomy and circuitry of nociception

Nociception is the biological processing of noxious stimuli. Encountering a noxious stimulus will trigger nociceptive signaling, but whether one ultimately perceives that as pain depends upon the circuits carrying these signals and how they are being regulated. In other words, nociception is not equivalent to pain. Consider, for instance, that many soldiers wounded in combat do not feel pain for some period, despite the severity of their injuries (Beecher, 1946). Although the wounded area is releasing signals of damage, a number of countervailing factors are likely in effect, such as the positive valence of the situation (an injury means the soldier is still alive and will soon depart the peril of battle) and the engagement of endogenous pain relief systems that inhibit the transmission of the nociceptive message to the brain (Basbaum & Fields, 1978; Melzack et al., 1982). Pain has two components, sensation and affect, and this example highlights that each is subject to modulation. Sensation is concerned with where, what, and how intense the stimulus is, but affect provides the imperative judgment of that stimulus being undesirable. Nociception must elicit both in order to produce pain.

Under normal conditions, nociception begins with a noxious stimulus that activates specialized receptors on the peripheral terminal of primary sensory neurons. For example, the cation channel TRPV1 is activated by capsaicin (the spicy compound in chili peppers), noxious heat, acidic conditions, and the endocannabinoid anandamide (Caterina et al., 1997; Tominaga et

al., 1998; Zygmunt et al., 1999), whereas the cation channel TRPA1 is activated by mustard oil, allicin (the pungent compound in garlic), and several other chemical irritants (Bandell et al., 2004; Bautista et al., 2005). The afferent nerve fibers that express these receptors can be grouped into two principal classes: Aδ and C (Woolf & Ma, 2007; Basbaum et al., 2009; Dubin & Patapoutian, 2010). Aδ fibers are thinly myelinated and small-to-medium diameter and C fibers are unmyelinated and small diameter. As they are myelinated, Aδ fibers have higher conduction velocities than C fibers and so generate the "first pain" (e.g., a pin prick) felt upon contact with a noxious stimulus. The slower C fibers are involved in the more long-lasting "second pain" (e.g., throbbing or aching) following the initial insult. Among the Aδ fibers, there are two further subclasses, type I and type II. Type I afferents respond to noxious mechanical stimulation and type II afferents respond to noxious heat. Similarly, there are multiple subclasses of C fibers, which include neurons that are chiefly heat- or mechano-sensitive (CH or CM) as well as those that are polymodal (CMH). The presence or lack of neuropeptides such as substance P and calcitonin gene-related peptide also divides the C fibers into peptidergic and non-peptidergic categories that differ in function. TRPV1-expressing fibers are mainly peptidergic and form a population of primary sensory neurons distinct from non-peptidergic fibers, many of which express the G-protein-coupled receptor MrgprD. Intriguingly, $TRPV1^+$ afferents mediate thermal nociception but not mechanical nociception, whereas the $MrgprD^+$ afferents mediate mechanical nociception but not thermal nociception (Cavanaugh et al., 2009).

As is the case for all primary afferents, neurons that innervate similar parts of the body cluster their cell bodies in a dorsal root ganglion (DRG) or for facial sensation, the trigeminal ganglion (TG). The peripheral axon of DRG and TG neurons is continuous with a central axon

that terminates in the spinal cord dorsal horn and caudal spinal trigeminal nucleus (also known as the medullary dorsal horn), respectively. The dorsal horn has a laminar structure and the individual classes of fibers have characteristic laminae that they target (Basbaum et al., 2009; Todd, 2010). Aδ fibers terminate in laminae I, II outer (II_o), and V, while peptidergic C fibers terminate in laminae I-II_o and non-peptidergic C fibers terminate in laminae II inner (II_i). All primary sensory neurons are excitatory, but the cells upon which they synapse in the dorsal horn may be excitatory or inhibitory. The majority of the latter cells are interneurons, which are engaged in local circuits. As any one cell can receive connections from numerous excitatory and inhibitory interneurons, signal transmission can be intricately facilitated or inhibited, allowing for precise control of nociception. Whether the modality specificity observed at the primary afferent level is maintained in the dorsal horn has been a long-standing question in the field of pain (Craig, 2003; Ma, 2010; Bráz et al., 2014). Many recent studies have used neurochemical markers to distinguish subsets of excitatory (Sun & Chen, 2007; Sun et al., 2009; Mishra & Hoon, 2013; Wang et al., 2013; Xu et al., 2013; Duan et al., 2014; Gutierrez-Mecinas et al., 2017) and inhibitory (Ross et al., 2010; Kardon et al., 2014; Bourane et al., 2015; Foster et al., 2015; Petitjean et al., 2015) interneurons and demonstrate their distinct behavioral functions in different modalities of pain as well as itch, a sensation closely related to pain that will be described later in this text. The four known subpopulations of inhibitory interneurons are defined by expression of neuropeptide Y, parvalbumin, galanin/dynorphin, or neuronal nitric oxide synthase. Markers for excitatory interneurons are not as well characterized, but include substance P, neurokinin B, neurotensin, and gastrin-releasing peptide (Todd, 2017).

Laminae I and V of the dorsal horn also contain excitatory neurons that project to the brain (Basbaum et al., 2009; Todd, 2010; Bráz et al., 2014). These projection neurons can receive nociceptive inputs directly from primary afferents or indirectly via interneurons. Unlike primary afferents and dorsal horn interneurons, there is presently only one known molecular marker for dorsal horn projection neurons, the neurokinin 1 receptor (NK1R), which is targeted by substance P. NK1R labels many of the lamina I projection neurons, and many of the lamina I projection neurons target the lateral parabrachial nucleus in the dorsolateral pons. From the lateral parabrachial nucleus are connections to the hypothalamus and amygdala, structures associated with the affective dimension of pain. Other dorsal horn projection neurons target the thalamus, from where the various thalamic nuclei send axons to a number of cortical areas. Some of these cortical areas, such as the primary and secondary somatosensory cortices, are involved in sensory-discriminative aspects of the pain experience, while others, such as the anterior cingulate cortex and the insular cortex, are implicated in pain-related affect. The activity of these diverse brain regions combines to form the perception of pain.

The role of sensitization in chronic pain

After an injury, the site of or surrounding the injury often becomes hypersensitive. Hypersensitivity can manifest as allodynia, in which formerly innocuous stimuli are now perceived as painful, and as hyperalgesia, in which noxious stimuli evoke more pain than they did prior to injury. During the healing process, allodynia and hyperalgesia are protective as they encourage one to exercise caution with the injured body part, preventing additional damage and enabling the injury to heal. Ideally, pain thresholds would return to baseline following the

recuperation period, but in some cases, the sensitized state persists and becomes pathological. This sensitization can occur at both the peripheral and central levels. In brief, peripheral sensitization stems from both a lowered threshold and a heightened excitatory drive from subsets of primary afferent nerve fibers. Tissue injury typically induces the release of a variety of inflammatory mediators, activating many signaling pathways and causing a host of changes including alterations in receptor expression and function, increased spontaneous firing, and activation of formerly "silent" C fibers (Hucho & Levine, 2007; Gold & Gebhart, 2010; Meacham et al., 2017). Central sensitization arises from heightened excitatory drive from central nervous system neurons and is generated by increased synaptic efficacy, shifts in descending controls, microglial activation, loss of inhibitory tone, and other events (Basbaum et al., 2009; Latremoliere & Woolf, 2009; Woolf, 2011; Meacham et al., 2017). The two major types of chronic pain, inflammatory pain (pain from tissue injury or inflammation) and neuropathic pain (pain following nerve injury), largely differ in terms of the mechanisms that promote sensitization as well as in the pharmacotherapies to which they respond (Xu & Yaksh, 2011). Because sensitization is at the root of chronic pain, mastering the specific conditions that initiate and maintain hyperexcitability in the DRG/TG and dorsal horn is the key to finding successful analgesic treatments.

Itch, a parallel to pain

In many respects, itch and pain are similar. Itch is defined as "an unpleasant sensation that elicits the desire to scratch" (Ikoma et al., 2006; Akiyama & Carstens, 2013; Bautista et al., 2014), sharing with pain a focus on the unpleasantness of the experience. As pain is produced by

nociception, so itch is the result of pruritoception. Also like nociception, pruritoception begins with the activation of receptors on the peripheral terminals of Aδ and C fibers. The H1 receptor responds to histamine, the classical pruritic stimulus, and is present on C fibers that express TRPV1 (Imamachi et al., 2009). There is also a histamine-independent itch pathway that has substantial clinical relevance due to the ineffectiveness of antihistamines in numerous chronic itch disorders. Non-histaminergic stimuli are transduced by various receptors, including members of the Mas-related G protein–coupled receptor (Mrgpr) family such as MrgprA3, which the antimalarial drug chloroquine binds to, and MrgprD, for which the amino acid β-alanine is a ligand (Liu et al., 2009; Liu et al., 2012). Interestingly, MrgprA3 is expressed on $TRPV1^{+}$, $H1R^{-}$ C fibers while MrgprD is expressed on afferents that do not overlap with the TRPV1 population (Dong et al., 2001; Zylka et al., 2005; Wilson et al. 2011).

That pruritoceptive and nociceptive receptors are on the same cell raises questions about the interaction of pain and itch. For example, knockout of TRPV1 in mice greatly diminishes scratching in response to histamine but not to other pruritogens (Shim et al., 2007; Imamachi et al., 2009), suggesting that function of this receptor contributes to histamine-dependent itch. However, as ablation of the entire TRPV1-expressing afferent causes a loss of itch behavior in response to a broad range of pruritogens (Imamachi et al., 2009; Liu et al., 2010; Kim et al., 2011; Wilson et al., 2013), it appears that the same sensory neurons that carry information about noxious heat also convey signals about itch-inducing stimuli. To provide ways for the system to differentiate the two sensations, sensory neurons or interneurons may release selective molecular mediators (e.g., gastrin related peptide and natriuretic polypeptide b are necessary for itch but not pain), change their firing patterns, or activate particular subsets of dorsal horn neurons (Bautista

et al., 2014). Evidence for inhibitory crosstalk between pain and itch also exists, such as when the itchiness of an insect bite is relieved by pinching the affected area (Ikoma et al., 2003). Of particular interest is the common observation, in humans and in mice, that while morphine can effectively relieve many pains, it frequently and concurrently provokes itch.

Like chronic pain, chronic itch can present as hypersensitivity. Hyperknesis is an elevated response to pruritic stimuli and alloknesis is a perception of itch in response to formerly innocuous stimuli, such as light touch. Pain and itch hypersensitivity also share features of their mechanisms. For instance, in normal animals, low threshold mechanical stimulation activates Aβ fibers and does not evoke pain or itch. Although the central terminals of Aβ fibers do form synapses with nociceptive/pruriceptive transmission neurons, under normal conditions, tonic inhibition by local interneurons blocks onward communication and serves to gate nociception/pruritoception. However, in chronic disease states, inhibitory interneurons exhibit reduced activity or may even die (Moore et al., 2002; Coull et al., 2003; Scholz et al., 2005; Ross et al., 2010; Akiyama et al., 2011), leading to disinhibition of the Aβ input to pain circuitry (Miraucourt et al., 2007; Bourane et al., 2015). Thus, a light brush on the skin may now generate sufficient input to dorsal horn projection neurons to create pain/itch. From this example and the extensive other similarities between pain and itch, it is clear that the same approaches used to study pain can also be applied to study itch. The circuits that underlie pain and itch behavior are highly complex, but the genetic, molecular, and electrophysiological profiles of the cells involved provide a valuable point of entry for inquiry as well as a wealth of potential therapeutic targets.

Sex differences in pain

Sex differences in pain processing have been repeatedly observed in both humans and experimental animals (Riley et al., 1998; Fillingim & Ness, 2000; Craft et al., 2004). Females demonstrate greater pain sensitivity in laboratory tests of nociception (Fillingim & Maixner, 1995), and strikingly, the prevalence of migraine, fibromyalgia, and many other painful disorders is significantly higher in women (Berkley, 1997; Unruh, 1996). There is also evidence to support a female predominance in itch sensitivity, both in rodents (Umeuchi et al., 2005; Green et al., 2006; Yamaura et al., 2014) and in humans (Lucey et al., 1986; Magerl et al., 1990; Młynek et al., 2009; Cassano et al., 2015). Differences in male and female behavior spring from the sex chromosomes, the main factor that distinguishes male from female zygotes. The sex chromosomes determine whether testes or ovaries will form, and in turn, those organs secrete distinct combinations of hormones that act organizationally during development and activationally to trigger behavior in the adult (Arnold, 2009). Thus, sex differences in the experience of pain and itch presumably have their roots in the three classes of gonadal steroid hormones: androgens (such as testosterone and dihydrotestosterone), progestogens (progesterone), and estrogens (the three most common being estrone, estradiol, and estriol) (Greenspan et al., 2007).

Estrogens and pain

Given the prevalence of chronic pain syndromes in women, estrogens are a pertinent and understandably important focus of pain research (Craft, 2007). Estrogens are synthesized by the

aromatization of testosterone or androstenedione, via the endoplasmic reticulum enzyme aromatase, a member of the cytochrome P450 family. The primary active estrogen is 17β-estradiol, which is produced by the ovaries, testes, adipose tissue, brain, and several other regions (Norman & Litwack, 1997). Estrogen receptor α (ERα) and estrogen receptor β (ERβ), the prototypical estrogen receptors (Maggiolini & Picard, 2010), have a fairly broad distribution as well, being most abundant in the reproductive system but also present elsewhere, including the lungs and brain (Couse et al., 1997; Kuiper et al., 1997). Not surprisingly, estrogens are therefore quite multifaceted, affecting mood, memory, movement, and much more, greatly extending their influence beyond their conventional functions in sexual development, mating, and pregnancy (McEwen & Alves, 1999). For example, aromatase can be detected in hippocampus and application of estradiol to hippocampal cultures both inhibits GABAergic interneurons and induces excitatory synapse formation (McEwen, 2002). That estradiol can affect synaptic plasticity has interesting implications for pain processing. As mentioned previously, following peripheral tissue or nerve injury, central circuits can develop persistent, heightened excitability through the process of central sensitization. With this enhanced signal transmission, conditions such as allodynia and hyperalgesia arise. Central sensitization derives from a number of activity- and transcription-dependent mechanisms (Ji et al., 2003), any of which could be regulated (or dysregulated) by estrogens via slow genomic and/or rapid nongenomic changes in cellular activity (Heldring et al., 2007).

The gonads are viewed as the essential source of estrogen, but aromatase expression is, in fact, driven by several different tissue-specific promoters and has been found in skin, bone, medial amygdala, hippocampus, and other areas (Nelson and Bulun, 2001; Simpson et al., 2002).

Neurosteroids refer to estrogens and other steroid hormones that are synthesized in the nervous system where they act to shape a wide variety of functions (Mellon & Griffin, 2002). In males and postmenopausal women, estrogens primarily act near their site of production in a paracrine, intracrine, or autocrine fashion (Lephart, 1996; Simpson, 2003). By contrast, reproductively able females experience fluctuating levels of estrogen across the menstrual/estrous cycle (e.g., blood concentrations of estrogen are high shortly before ovulation but can fall to male-like levels at other stages; Craft et al., 2004; Becker et al., 2005), so it is possible that there are times when circulating estrogens predominate and times when local estrogens are more effective. Taken together, local estrogen synthesis may therefore impact males and females differently, potentially spurring sex differences in pain.

Numerous studies testing the systemic effects of estrogens on pain have unfortunately yielded results that often contradict one another (Craft, 2007). This lack of agreement stems, in part, from the fact that these diverse studies compare assays that affect different qualities (e.g., visceral vs. cutaneous, thermal vs. chemical), monitor diverse responses (e.g., flinching, licking, stretching), and use animals that have undergone very different hormonal manipulations (e.g., intact vs. gonadectomized, gonadectomized with hormone replacement vs. gonadectomized without hormone replacement) (Fillingim & Ness, 2000; Kuba & Quinones-Jenab, 2005). As pain and itch research has converged on the discrete molecules, cells, and circuits controlling nociception and pruritoception, so, too, must our investigation of estrogen's contributions to pain and itch (Amandusson & Blomqvist, 2013). Accordingly, the work that comprises this dissertation examines the spinal cord circuits through which estrogen can influence pain and itch processing in two ways. The first approaches the question of the source of estrogen and the

opportunity for local estrogen synthesis through molecular characterization of cells in the medullary and spinal dorsal horns that express aromatase. The second concerns the site of estrogenic action, the receptor, by assessing the behavioral consequences of ablating ERα-expressing neurons in the spinal cord dorsal horn.

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CHAPTER 2

Neuronal aromatase expression in pain processing regions of the medullary and spinal cord dorsal horn

2-1. ABSTRACT

In both acute and chronic pain conditions, women tend to be more sensitive than men. This sex difference may be regulated by estrogens, such as estradiol, that are synthesized in the spinal cord and brainstem and act locally to influence pain processing. To identify a potential cellular source of local estrogen, here we examined the expression of aromatase, the enzyme that catalyzes the conversion of testosterone to estradiol. Our studies focused on primary afferent neurons and on their central targets in the spinal cord and medulla as well as in the nucleus of the solitary tract, the target of nodose ganglion-derived visceral afferents. Immunohistochemical staining in an aromatase reporter mouse revealed that many neurons in laminae I and V of the spinal cord dorsal horn and caudal spinal trigeminal nucleus and in the nucleus of the solitary tract express aromatase. The great majority of these cells also express inhibitory interneuron markers. We did not find sex differences in aromatase expression and neither the pattern nor the number of neurons changed in a sciatic nerve transection model of neuropathic pain or in the Complete Freund's adjuvant model of inflammatory pain. A few aromatase neurons express Fos after cheek injection of capsaicin, formalin, or chloroquine. In total, given their location, these aromatase neurons are poised to engage nociceptive circuits, whether it be through local estrogen synthesis or inhibitory neurotransmitter release.

2-2. INTRODUCTION

The great majority of clinical pain conditions predominate in women (Unruh, 1996; Berkeley, 1997; Mogil, 2012). Women also exhibit lower pain thresholds and tolerances to a variety of noxious stimuli, which suggests that females are more sensitive to painful stimuli, even at baseline (Fillingim & Maixner, 1995; Riley et al., 1998; Mogil, 2012). As sex hormones are likely to facilitate these sex differences, particular attention has been paid to the contribution of estrogen in nociception (Craft et al., 2004; Kuba & Quinones-Jenab, 2005; Aloisi & Bonifazi, 2006). Unfortunately, because of the broad distribution of estrogen receptors in neural and non-neural tissues as well as the wide range of effects linked to estrogen, determining unequivocally whether estrogen is selectively pro- or anti-nociceptive has proven difficult (Craft, 2007). For this reason, a more detailed analysis of the existence and function of estrogen and its receptors in discrete cell populations implicated in pain processing is essential (Amandusson & Blomqvist, 2013).

Although the gonads are the primary source of estrogen circulating in the blood, there is now considerable evidence that numerous other tissues, including skin, fat, and brain, produce estrogen that acts in or near those locations (McEwen & Alves, 1999; Simpson, 2003). In fact, several groups have proposed that there is estrogen synthesis in the spinal cord. For example, Evrard et al. (2000) detected aromatase, the enzyme that converts androgens to estrogens, in the spinal cord dorsal horn of Japanese quails and Zhang, Xiao, et al. (2012) demonstrated electrically evoked estrogen release in rat spinal cord slices. Furthermore, in both quails and rats, the behavioral consequences of manipulating spinal estrogen levels by administering estrogens

or aromatase inhibitors suggests that spinal cord-derived estrogen has pro-nociceptive effects (Evrard & Balthazart, 2004; Zhang, Lü, et al., 2012).

The earlier work that characterized the distribution of aromatase used antibodies directed against the protein. Because of significant concerns with antibody specificity, here we took advantage of a transgenic mouse line in which an internal ribosome entry site coupled to a nuclear lacZ reporter has been knocked into the aromatase gene (Wu et al., 2009), allowing for very precise molecular mapping of the distribution of aromatase-expressing cells. We also characterized the cells based on co-expression of a host of molecular markers and examined the extent to which these cells are influenced in different mouse models of acute and chronic pain.

2-3. METHODS

Mouse lines

We used aromatase IRES-PLAP-IRES-nlacZ reporter mice (Wu et al., 2009; homozygous for reporter allele, RRID:MGI:4430066 and heterozygous for reporter allele, RRID:MGI:5634564). Animals were 8-12 weeks old at time of perfusion. All experiments were performed in accordance with the University of California, San Francisco's Institutional Animal Care and Use Committee guidelines.

Immunohistochemistry

Animals were deeply anesthetized using 250-400 mg/kg 2,2,2-Tribromoethanol (Avertin, Sigma-Aldrich, St. Louis, Missouri) and then transcardially perfused with 10 ml of phosphate-buffered saline (PBS) followed by 30 ml of 4% formaldehyde in PBS (37% formaldehyde diluted 1/10; ACROS Organics, Morris Plains, New Jersey). All perfusions and incubations used 1X PBS with the exception of those involving staining for sst2a, which required PBS containing 300 mM NaCl rather than the typical concentration of 137 mM NaCl. Dorsal root ganglia (DRGs), trigeminal ganglia (TGs), spinal cord, and brain were dissected out and post-fixed in 4% formaldehyde in PBS for 3-6 hours at room temperature. Tissue was cryo-protected in 30% sucrose for at least one night and then sectioned on a cryostat; spinal cord and medulla were cut at a thickness of 25 μm and 35 μm, respectively, and collected in PBS while DRG and TG were cut at 14 μm and directly mounted onto slides. For staining, tissue was blocked for one hour in

10% normal goat serum in PBS containing 0.3% Triton X-100 and then incubated overnight at room temperature in primary antibodies diluted in 1% normal goat serum in PBS containing 0.3% Triton X-100. Primary antibodies used are as indicated in Table 1. Following overnight incubation, tissue was washed three times with PBS and then incubated with secondary antibodies diluted 1:1000 in PBS for a minimum of two hours at room temperature. Secondary antibodies were all Alexa Fluor 488, 546, 594, or 647-conjugated (Thermo Fisher Scientific, Waltham, Massachusetts) and raised in goat against the appropriate primary species. After several PBS washes, tissue was mounted onto slides (if necessary) and allowed to briefly dry before coverslipping with Fluoromount-G aqueous mounting medium (SouthernBiotech, Birmingham, Alabama).

Antibody characterization

Table 1. Primary antibodies

Antibody	**Immunogen**	**Source**	**Concentration**
beta Galactosidase (β-gal)	Purified full-length native protein from E. coli	Abcam, Cat# ab9361; RRID:AB_307210; chicken, polyclonal	1:10,000
NeuN	Purified mouse brain cell nuclei, clone A60	Millipore, Cat# MAB377; RRID:AB_2298772; mouse, monoclonal	1:5000
Fluorogold	Glutaraldehyde-conjugated Fluoro-Gold	Protos Biotech, Cat# NM-101 FluGgp; RRID:AB_2314409; guinea pig, polyclonal	1:1000
TRPV1	C-terminus 15aa (EDAEVFKDSMAPGEK) of mouse transient receptor potential vanilloid subtype 1	D. Julius, University of California, San Francisco; guinea pig, polyclonal	1:5000

Antibody	Immunogen	Source	Concentration
Pax2	Synthetic peptide within human paired box 2 protein, aa1-20 at N-terminus, clone EP3251	Abcam, Cat# ab79389; RRID:AB_1603338; rabbit, monoclonal	1:4000
sst2a	C-terminus 15aa (ETQRTLLNGDLQTSI) of mouse somatostatin receptor subtype 2A	Gramsch Laboratories, Cat# SS-870; RRID:AB_2491104; guinea pig, polyclonal	1:10,000
Lmx1b	Full-length LIM homeobox transcription factor 1 beta protein from mouse	T. Müller and C. Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany; RRID:AB_2314752; guinea pig, polyclonal	1:10,000
ERα	15aa (TYYIPPEAEGFPNTI) at C-terminus of rat estrogen receptor α	Millipore, Cat# 06-935; RRID:AB_310305; rabbit, polyclonal	1:10,000
Fos	Synthetic peptide within human c-Fos, aa4-17 (SGFNADYEASSSRC), Ab-5	Calbiochem, Cat# PC38; RRID:AB_2106755; rabbit, polyclonal	1:5000
ATF3	C-terminus 19aa (PEDERNLFIQQIKEGTLQ S) of human activating transcription factor 3	Santa Cruz Biotechnology, Cat# sc-188; RRID:AB_2258513; rabbit, polyclonal	1:2000
Iba1	Synthetic peptide corresponding to C-terminus 14aa (PTGPPAKKAISELP) of ionized calcium-binding adapter molecule 1	Wako, Cat# 019-19741; RRID:AB_839504; rabbit, polyclonal	1:1000

Chicken anti β-gal (Abcam, Cat# ab9361, RRID:AB_307210) does not produce staining in wild-type animals, i.e. animals that do not express the aromatase reporter allele (unpublished observation).

Mouse anti NeuN (Millipore, Cat# MAB377, RRID:AB_2298772) recognizes neuronal nuclei and cytoplasm. Antibody specificity has been evaluated with immunohistochemistry and

immunoblot analysis, showing that immunoreactivity is present in neurons and in nervous tissue but not in glia or in other organs (Mullen et al., 1992).

Guinea pig anti Fluorogold (Protos Biotech, Cat# NM-101 FluGgp, RRID:AB_2314409) signal completely overlaps with Fluoro-Gold fluorescence observed with ultraviolet illumination (data not shown; Al-Khater & Todd, 2009). Specificity was also confirmed through a lack of immunoreactivity following preincubation with Fluoro-Gold (manufacturer's information). Furthermore, there is no Fluorogold staining in animals that were not injected with Fluoro-Gold.

Rabbit anti Pax2 (Abcam, Cat# ab79389, RRID:AB_1603338) staining pattern in this study is in agreement with previous reports that characterize spinal cord dorsal horn Pax2-expressing cells as inhibitory interneurons (Kardon et al., 2014; Punnakkal et al., 2014). In addition, for this particular antibody, Western blot from human fetal kidney tissue recognizes a band at the proper expected size of 45 kDa (manufacturer's information). Furthermore, immunostaining in aromatase reporter mice that were crossed to a GAD67-GFP reporter line indicate that around 80% of the aromatase cells are GABAergic, which is similar to the percentage of aromatase cells that are Pax2-expressing (data not shown).

Guinea pig anti sst2a (Gramsch Laboratories, Cat# SS-870, RRID:AB_2491104) has been previously shown to label inhibitory neurons in the rodent spinal cord dorsal horn (Todd et al., 1998) and in a pattern that matches other reports of sst2a expression (Holloway et al., 1996; Schindler et al., 1997). Specificity of this antibody has been confirmed through dot-blot assays demonstrating detection of sst2a but not somatostatin receptors sst1, sst2b, or sst3; Western blots

from rat brain tissue displaying a broad band of suitable size (80 kDa); and lack of immunoreactive cells following preadsorption with the immunizing peptide (Schulz et al., 1998). Antibody was kindly provided by Andrew J. Todd, University of Glasgow.

Guinea pig anti Lmx1b (gift of T. Müller and C. Birchmeier, Max-Delbrück-Center for Molecular Medicine; Berlin, Germany, RRID:AB_2314752) specifically marks excitatory dorsal horn interneurons of the dI5 and dILB lineages (Yang et al., 2010). The staining pattern in our report is consistent with previous articles (Del Barrio et al., 2013; Szabo et al., 2015).

Rabbit anti ERα (Millipore, Cat# 06-935, RRID:AB_310305) detects in Western blots a roughly 58 kDa band from MCF7 cell lysate (manufacturer's information) and a 55 kDa band from cichlid whole brain extract (Munchrath & Hofmann, 2010). Preincubation with the antigen eliminates all bands (Friend et al., 1997). In addition, we detected no signal in immunostainings of spinal cord tissue from ERα conditional knockout mice (unpublished observation).

Rabbit anti c-Fos (Calbiochem, Cat# PC38, RRID:AB_2106755) labels c-Fos without cross-reactivity with other Fos-related antigens in the rat central nervous system (Hoffman et al., 1992; Rinaman et al., 1993). In tests by the manufacturer, the antibody was able to bind to c-Fos and v-Fos (55 and 62 kDa, respectively) but not Jun (39 kDa).

Guinea pig anti TRPV1 (gift of David Julius, University of California, San Francisco) is being used in this study as a marker of primary afferents in the DRG. Staining from this antibody matches previous reports and importantly, there is no antibody staining in tissue from TRPV1

knockout mice (Bráz & Basbaum, 2010) or in the spinal cord of mice in which TRPV1 central terminals have been ablated (Cavanaugh et al., 2009).

Rabbit anti ATF3 (Santa Cruz Biotechnology, Cat# sc-188, RRID:AB_2258513) is a well characterized antibody that labels injured DRG neurons (Bráz et al., 2011; Starkey et al., 2009). Western blot from rat brain tissue produces an appropriate band approximately 21 kDa in size (Yamanaka et al., 2011). Our data show that ATF3 is only induced on the nerve-injured side, which is in agreement with previous studies (Tsujino et al., 2000; Bráz & Basbaum, 2010; Guan et al., 2016).

Rabbit anti Iba1 (Wako, Cat# 019-19741, RRID:AB_839504) has been used extensively as a microglia marker and the spinal cord staining pattern seen in our data is consistent with other reports (Pineau & Lacroix, 2007; Yamanaka et al., 2011; Guan et al., 2016). This antibody has been demonstrated to be specific to microglia as Western blotting displays a single band of correct expected size of 17 kDa only in microglia-containing tissue samples and immunohistochemistry detects no signal in astrocytes, oligodendrocytes, or neurons (manufacturer's information; Imai et al., 1996; Ito et al., 1998; Yamanaka et al., 2011).

Retrograde tracing

Under intraperitoneal 80-100 mg/kg ketamine + 5-10 mg/kg xylazine anesthesia, mice were placed in stereotaxic apparatus (Kopf Instruments, Tujunga, California) and 0.5-1 µl of either Fluoro-Gold (Fluorochrome, Denver, Colorado) or red RetroBeads (Lumafluor, Durham,

North Carolina) was injected into the left lateral parabrachial nucleus. Lateral parabrachial nucleus was located according to coordinates from Paxinos and Franklin's *The Mouse Brain in Stereotaxic Coordinates*. Animals were perfused 3-9 days later and tissue was processed for immunohistochemistry.

Fos induction

Capsaicin (Sigma-Aldrich; 5 µg in 30 µl saline with 10% Tween-80 and 10% ethanol for cheek, 3 µg in 10 µl saline with 10% Tween-80 and 10% ethanol for hindpaw), 2% formalin (37% by weight formaldehyde, diluted 1/50 in saline; 50 ul for cheek, 10 µl for hindpaw), or chloroquine (chloroquine diphosphate salt, Sigma-Aldrich; 200 µg in 50 µl saline for cheek, 40 µg in 20 µl saline for hindpaw) was injected into the left cheek (shaved the day before injection) or the plantar surface of the left hindpaw of mice that were lightly restrained with a towel. 90 minutes later, mice were perfused and tissue was processed for immunohistochemistry as above.

Chronic injury models

For infraorbital or sciatic nerve transection, mice were anesthetized in the same manner as they were for retrograde tracing experiments. The left cheek or left hind leg was shaved, a small incision was made in the whisker pad area or thigh, and then the appropriate nerve was exposed. Following the cutting of the nerve (and in the case of sciatic nerve transection, excision of 2 mm of nerve), cheek or leg was sutured and mice were allowed to recover from anesthesia. One week later, mice were perfused and tissue was processed for immunohistochemistry. For

Complete Freund's adjuvant (CFA) injections, mice were lightly restrained with a towel and 20 µl of CFA (Sigma-Aldrich; 1:1 emulsion in saline) was injected into the left cheek or the plantar surface of the left hindpaw. Three days later, mice were perfused and tissue was processed for immunohistochemistry.

Confocal and epifluorescent imaging

All images except medulla images were taken on a LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) equipped with 405 nm, 488 nm, 555 nm, and 639 nm diode lasers, a main dichroic beam splitter URGB and a gradient secondary beam splitter for LSM 700 using a 10× EC Plan-Neofluar (10×/0.3) for sagittal spinal cord sections or a 20× Plan-Apochromat (20×/0.8) objective (Zeiss). Image acquisition was done with ZEN 2010 (Zeiss), and image dimensions were 1024 × 1024 pixels with an image depth of 12 bits. Two times averaging was applied during image acquisition. Laser power and gain were adjusted to avoid saturation of single pixels and kept constant for each experiment. Medulla images were taken on an Axioimager M2 (Zeiss) equipped with AF488, AF568, Cy5, and DAPI filter sets and an Axiocam 506 mono camera using a 20× Plan-Apochromat (20×/0.8) objective (Zeiss) in the "Tiling mode" of Zen2 Pro (Zeiss). Image acquisition was performed with fixed exposure times for each channel and with a 10% overlap of neighboring images. Stitching was done in Zen2 Pro based on the NeuN channel using the "stitching/fuse tiles" function. Adjustment of brightness/contrast, changing of artificial colors (LUT), and maximum projections of Z-stack images were done in Fiji/ImageJ (https://fiji.sc, RRID:SCR_002285). All images of the same experiment were processed in an identical manner. For images in Figures 3, 7, and 8, the

"Remove Outliers" filter in Fiji/ImageJ was applied to digitally remove artifacts and debris in areas outside of the tissue. This filter was set to sample the value of pixels in a 100-pixel radius and replace pixels that were more than 10 units brighter than the median with the median value.

Quantification of double-labeled cells

For counting cells in the spinal cord, the dorsal horn of 4-6 randomly selected spinal cord sections from each mouse was imaged using identical imaging parameters. Labeled cells in each channel were identified using the Isodata Threshold algorithm in Fiji/ImageJ, and counted with the "Particle Analyzer" function with a size range of 15-150 μm and a circularity of 0.5-1. An overlay of both masks was created to identify double-labeled cells in each section. For counting cells in the caudal spinal trigeminal nucleus (cSTN) and nucleus of the solitary tract (NST), the entirety of 3-4 randomly selected medulla sections from each mouse was imaged using identical imaging parameters. In the resulting stitched images, cSTN and NST were distinguished based on typical area morphology from Paxinos and Franklin's mouse brain atlas. Labeled cells in each channel were manually counted in Fiji/ImageJ using the "Cell Counter" tool. Double-labeled cells were identified by color change; an overlap of cyan and magenta to produce white indicated that a cell was double-labeled. For both spinal cord and medulla, the percentage of double-labeled cells was calculated for each mouse as the total number of double-labeled cells divided by the total number of β-gal positive cells from the sections used for quantification. Final percentages are displayed as the averages of both male and female mice as there was no statistically significant difference in overlap between the sexes as determined by Student's *t*-test.

Statistical analysis

Unpaired (Student's) *t*-tests and two-way ANOVA with Tukey's test to correct for multiple comparisons were run using GraphPad Prism (version 6.0e, https://www.graphpad.com/scientific-software/prism, RRID:SCR_002798). Tests are as indicated in figure legends.

2-4. RESULTS

Aromatase expression in spinal cord and medulla

Immunostaining for β-galactosidase (β-gal, the product encoded by lacZ) in the aromatase reporter mouse revealed an extensive distribution of β-gal immunoreactivity that overlapped remarkably with regions of the spinal cord and brainstem that process nociceptive information (Figs. 1 and 2). In the medulla, chains of β-gal$^+$ nuclei are visible in laminae I and V of caudal spinal trigeminal nucleus (cSTN; Fig. 1a), an area that receives nociceptive input from primary sensory neurons of the trigeminal ganglion (TG; Price, Dubner, & Hu, 1976; Robertson & Arvidsson, 1985). We observed a comparable pattern, although with many fewer cells, in laminae I and V of the spinal cord dorsal horn (Fig. 2a-b), which receives nociceptive input from primary sensory neurons of the dorsal root ganglion (DRG; Adrian, 1928; Foerster, 1933). Interestingly, we also found scattered aromatase-positive cells in the nucleus of the solitary tract (NST; Fig. 1A), which receives input from nodose ganglion-derived visceral afferents (Contreras et al., 1992). Every β-gal$^+$ cell co-labeled with the neuronal marker NeuN, indicating that these aromatase-expressing cells are neurons (Figs. 1a and 2b, insets 1-2). In contrast, we never found β-gal-immunoreactive cells in the aromatase reporter mouse in either TG or DRG (Figs. 1b and 2c), despite extensive estrogen receptor expression in these sensory ganglia (Sohrabji et al., 1994; Papka et al., 1997; Taleghany et al., 1999; Papka et al., 2001). Similarly, although the aromatase-expressing cells are found in close proximity to estrogen receptor α (ERα)-expressing neurons, we found no evidence for overlap in the medulla or spinal cord (Fig. 3a-b).

Quantification of aromatase expression in males and females

As a sex difference in aromatase expression in the brain impacts sexually dimorphic behaviors (Wu et al., 2009; Unger et al., 2015), we next asked if there are different numbers of β-gal$^+$ cells in the spinal cord and medulla of male and female mice, a difference that might underlie sexual dimorphisms in pain processing. cSTN and NST display no sex differences (Fig. 4a; unpaired t-test – cSTN, p = 0.84, male: 54.6 ± 5.9 and female: 56.1 ± 4.8 cells per section ± SEM; NST, p = 0.18, male: 10.5 ± 0.60 and female: 8.9 ± 0.85 cells per section ± SEM). Across the cervical, thoracic, lumbar, and sacral regions of the spinal cord, we found neither a significant difference between males and females nor a difference in numbers of cells at different segmental levels (Fig. 4b; two-way ANOVA – sex effect: $F_{(1,41)} = 0.031$, $p = 0.86$; region effect: $F_{(3,41)} = 0.17$, $p = 0.92$; interaction: $F_{(3,41)} = 0.40$, $p = 0.75$; overall male: 7.0 ± 0.26 cells per section ± SEM, overall female: 7.1 ± 0.38 cells per section ± SEM; see Fig. 4 legend for breakdown by spinal region).

Overlap of aromatase and markers of projection neurons and interneurons

Laminae I and V contain major populations of nociceptive projection neurons (Todd et al., 2000; Price et al., 2003; Klop et al., 2005; Bráz et al., 2014), with the great majority (~85%) of neurons targeting the parabrachial nucleus of the rostral pons (Hylden et al., 1989; Spike et al., 2003). Because of the striking concentration of the aromatase-expressing cells in laminae I and V, we injected retrograde tracers into one side of the lateral parabrachial nucleus of reporter mice and double-labeled tissue to identify aromatase-expressing projection neurons. Although we

observed many projection neurons neighboring cells with β-gal$^+$ nuclei, we never detected double-labeled cells (Fig. 5a-c). Based on this finding, we conclude that aromatase-expressing neurons do not project, but rather are interneurons that predominate in laminae I and V.

Dorsal horn interneurons consist of both excitatory and inhibitory interneurons and within these general populations are neurochemically distinct subtypes. We first co-stained the aromatase cells with an antibody that recognizes Pax2, a selective marker of dorsal horn inhibitory interneurons (Del Barrio et al., 2013; Punnakkal et al., 2014). Fig. 6a,b,e show that ~80% of the aromatase-expressing neurons in both the spinal cord and cSTN are Pax2-expressing and thus inhibitory. We next examined the subtypes of inhibitory interneurons, which Todd and colleagues (2010) defined by their differential expression of galanin, neuropeptide Y, neuronal nitric oxide synthase, and parvalbumin. The somatostatin receptor subtype 2A (sst_{2A}) marks about 50% of spinal inhibitory interneurons and encompasses the galanin, neuronal nitric oxide synthase, and some of the neuropeptide Y populations (Polgár, Durrieux et al., 2013; Polgár, Sardella, et al., 2013). Somewhat unexpectedly, we found no overlap of sst_{2A} and β-gal in the spinal cord (Fig. 6c; 0 sst_{2A}^+ in 130 β-gal$^+$ cells, n = 2 males, 2 females), which demonstrates that the aromatase-expressing cells belong to the sst_{2A}-negative class of inhibitory interneurons. Finally, because a small number of aromatase-expressing cells were Pax2-negative, we also immunostatined the cells for Lmx1b, a marker for spinal excitatory neurons (Del Barrio et al., 2013; Szabo et al., 2015). As expected we identified occasional Lmx1b-β-gal double-labeled cells (Fig. 6d,e). Based on these findings, we conclude that the great majority of aromatase-expressing neurons are inhibitory and of the sst2a receptor-negative subtype.

Algogen- and pruritogen-induced Fos expression

Various types of noxious stimulation induce expression of the immediate early gene *Fos*, prompting the production of the Fos protein in activated dorsal horn neurons (Hunt et al., 1987; Menétrey et al., 1989). As different stimuli give rise to different patterns of Fos expression, co-localization with Fos could provide insights into whether the aromatase-expressing cells respond to specific pain modalities. Fig. 7a-c illustrates examples of β-gal^{+} cells in the medullary dorsal horn that overlap with Fos induced by the injection of the algogens capsaicin and formalin or the pruritogen chloroquine. When quantified, we found that capsaicin induced Fos in 17.6% of cSTN aromatase neurons, formalin in 12.1% of these cells, and chloroquine in 13.9%. In contrast, the same agents injected into the hindpaw did not induce any overlap in the spinal cord dorsal horn (Fig. 7d and data not shown).

Aromatase expression in models of chronic neuropathic or inflammatory pain

Two previous studies reported that nerve injury results in increased aromatase expression and estrogen synthesis in DRG neurons and in the spinal cord dorsal horn (Ghorbanpoor et al., 2014; Schaeffer et al., 2010). Here we sought to confirm those observations by assessing aromatase expression in the TG or DRG and cSTN or spinal cord after a complete unilateral transection of the infraorbital or sciatic nerves. We performed these studies in both male and female aromatase reporter mice and monitored β-gal expression 7-days post injury, a time point when rodents show behaviors indicative of spontaneous pain and significant upregulation of neurochemical markers of nerve injury (Basbaum, 1974; Wall et al., 1979; Coderre et al., 1986;

Villar et al., 1989; Bráz et al., 2011). As expected, we recorded activating transcription factor 3 (ATF3), a marker of injured sensory neurons (Tsujino et al., 2000; Bráz & Basbaum, 2010), in large numbers of TG and L4/L5 DRG neurons ipsilateral to the transection (Fig. 8a and 8c, upper right). However, we never detected β-gal signal in either the ipsilateral or contralateral DRG (Fig. 8a and 8c, lower left and right). In a similar fashion, ionized calcium binding adaptor molecule 1 (Iba1)-expressing spinal cord microglia are readily apparent after nerve transection (Fig. 8b and 8d, upper right; Ito et al., 1998; Romero-Sandoval et al., 2008), but the β-gal expression pattern did not change (Fig. 8b and 8d, lower left and right). Finally, in a separate set of experiments we evaluated aromatase expression under conditions of prolonged inflammation by injecting Complete Freund's adjuvant (CFA) into the hindpaw. At 3 days post-injection when animals demonstrate profound thermal and mechanical hypersensitivity (Ma & Woolf, 1996; Malmberg et al., 2003), we again found no changes in the number of β-gal$^+$ cells in the DRG or spinal cord dorsal horn (data not shown). Finally, in neither of these chronic pain models did we observe sex differences in aromatase expression in either DRG or spinal cord (data not shown). Taken together, our data indicate that neither peripheral nerve injury nor inflammation modifies aromatase expression in the spinal cord or DRG at the time points examined.

2-5. DISCUSSION

In the present study, we identified and characterized a subpopulation of spinal and medullary neurons that express aromatase, a critical enzyme for estrogen synthesis. The aromatase-expressing cells are concentrated in laminae I and V of the spinal cord dorsal horn and in its trigeminal homologue, the caudal spinal trigeminal nucleus, as well as in the nucleus of the solitary tract. The spinal cord dorsal horn processes somatic sensory information, the caudal spinal trigeminal nucleus processes facial sensory information, and the nucleus of the solitary tract processes visceral sensory information. The great majority of aromatase-expressing cells are inhibitory interneurons, but appear not to express markers of the major inhibitory interneuron subpopulations. As some of these neurons express Fos in response to a variety of proalgesic or pruritic agents, it is likely that they are engaged in the setting of acute pain and itch. In contrast, their contribution in chronic pain models is unclear, given that we found no difference in the number or pattern of aromatase expressing neurons in several models of nerve or tissue injury. Finally, across the tissues and conditions examined, we found no difference in numbers of aromatase neurons in male and female mice.

Studies of aromatase expression and technical considerations

Aromatase is a member of the very large and structurally similar cytochrome P450 superfamily (Danielson, 2002). As a result, it can be difficult to generate antibodies that selectively recognize aromatase without crossreacting with any of the other 101 cytochrome P450 genes in the mouse (Nelson et al., 2004). In fact, this potential limitation of an

immunocytochemical approach is what motivated several groups to develop reporter mouse lines (Wu et al., 2009; Stanić et al., 2014). In our experiments, we used the mouse generated by Wu and colleagues, which drives β-galactosidase expression under the control of aromatase promoters without disrupting normal aromatase function. Importantly, β-gal immunostaining in this reporter mouse successfully recapitulates the brain aromatase *in situ* hybridization pattern (Wu et al., 2009; Unger et al., 2015).

Our results using the aromatase reporter mouse are largely consistent with previous immunocytochemical reports of aromatase expression in the medullary and spinal cord dorsal horns in the Japanese quail, rat, and mouse (Horvath & Wikler, 1999; Evrard et al., 2000; O'Brien et al., 2015; Smeester et al., 2016). On the other hand, although our finding that these cells are indeed neurons agrees with the conclusion of some groups (Horvath & Wikler, 1999; Evrard et al., 2000), it is at odds with another group that concluded that aromatase expression arises from astrocytes (O'Brien et al., 2015; Smeester et al., 2016). Furthermore, our finding of no expression of aromatase in sensory neurons contrasts significantly with the report of Schaeffer et al. (2010) that described aromatase immunoreactivity in DRG neurons using a mouse monoclonal antibody.

Neuronal estrogen and pain processing

Aromatase catalyzes the final steps that convert androgens, such as testosterone, to estrogens, such as estradiol. The presence of aromatase expression in spinal and medullary neurons thus indicates that these neurons are capable of synthesizing estrogens. As both the

spinal and medullary dorsal horns express estrogen receptors (Amandusson et al., 1995; Shughrue et al., 1997; Merchenthaler et al., 2004; Bereiter et al., 2005; Vanderhorst et al., 2005; Dun et al., 2009), the aromatase cells in laminae I and V are ideally positioned to release estrogen in circuits that process nociceptive/pain messages. Given the broad distribution of estrogen receptor-expressing neurons in the superficial dorsal horn, the estrogen receptors may be located in the same cell (autocrine and intracrine signaling), in cells in the vicinity (paracrine signaling), or even in synaptic partners (synaptocrine signaling, see Remage-Healey et al., 2011 for review). Our results showing that ERα is expressed by cells adjacent to but not in the aromatase neurons indicate that paracrine and synaptocrine signaling are more likely. Co-staining for other estrogen receptors as well as circuit tracing in the aromatase reporter mouse should provide valuable information about the targets of local estrogen.

Despite the abundance of superficial dorsal horn interneurons that express estrogen receptors, there is surprisingly little information as to the function of local estrogen synthesis in nociception. When applied to neurons, estrogen increases intrinsic excitability and excitatory transmission (Woolley, 2007), which suggests that local estrogen would be pronociceptive, promoting sensitization of circuits and subsequent heightened responses to painful stimuli. Indeed, for acute pain, estrogen synthesized in the spinal cord appears to be pronociceptive (Evrard & Balthazart, 2004; Zhang, Lü, et al., 2012; Zhang, Xiao, et al., 2012). Injection of estrogen into the paw, which would mimic the effects of locally synthesized estrogen acting on primary afferents, also generates hyperalgesia (Hucho et al., 2006; Kuhn et al., 2008). In contrast, there is evidence that sensory neuron-derived estrogen is antinociceptive (Fusi et al., 2014). There is also a lack of agreement as to estrogen's function in chronic pain models. It is of

interest, for example, that dorsal horn ERα-expressing neurons have the ability to synthesize enkephalin, an endogenous opioid peptide, and that its precursor, preproenkephalin, is acutely upregulated by estrogen (Amandusson et al., 1996; Amandusson et al., 1999). As such, estrogen synthesized by medullary and spinal aromatase-expressing cells could activate ERα-expressing neurons to engage the endogenous analgesic system. On the other hand, although antinociceptive effects of primary afferent and spinal cord-derived estrogen were demonstrated in two neuropathic pain models (Schaeffer et al., 2010; Ghorbanpoor et al., 2014), a different group observed a pronociceptive effect in a bone cancer pain model (O'Brien et al., 2015; Smeester et al., 2016). Surprisingly, although aromatase expression was increased in each of these chronic pain models, using the reporter mouse we found no changes after nerve or tissue injury. Although these results are seemingly contradictory, the effects of estrogen are heavily dependent on the anatomical location, the type of pain that is assessed, and a host of other factors, making direct comparison of studies difficult (Craft, 2007Amandusson & Blomqvist, 2013).

The contribution of local estrogen to pain processing may also depend on the sex of the animal studied. In the rodent brain, aromatase neurons exhibit differential expression in males and females and contribute to sexually dimorphic behaviors, in part through the action of locally synthesized estrogen (Wu et al., 2009; Unger et al., 2015). Surprisingly, in spinal cord and medulla, comparing males and females, we found no statistical difference in numbers of aromatase-expressing cells. We appreciate, however, that numbers of cells may not be the critical contributor. For example, it is possible that these cells are more active in one sex compared to the other. Notably, males have a greater amount of circulating testosterone compared to females,

which raises the possibility that the aromatase neurons in males are more likely to encounter testosterone that can be aromatized into estrogen. Consequently, although females have overall higher systemic levels of estrogen, males could experience greater local concentrations of estrogen that act more discretely on nearby nociceptive circuits. Studies that measure local estrogen in males and females, perhaps by microdialysis, would be useful to test this hypothesis.

Aromatase as an inhibitory cell marker in the spinal cord and medulla

In addition to being a source of estrogen, these cells likely release neurotransmitters that modify nociceptive circuits. Through our retrograde tracing and immunohistochemical co-labeling experiments, we found that the majority of the aromatase-expressing cells express γ-aminobutyric acid (GABA; data not shown) and the Pax2 transcription factor, markers of inhibitory interneurons. On the other hand, we failed to define the subtype of inhibitory GABAergic interneuron, four of which have been demonstrated to date: neuronal nitric oxide synthase, galanin, neuropeptide Y, and parvalbumin (Laing et al., 1994; Sardella, Polgár, Garzillo, et al., 2011; Sardella, Polgár, Watanabe, & Todd, 2011; Tiong et al., 2011). Furthermore, although almost fifty percent of dorsal horn inhibitory interneurons express the sst_{2A} subtype of somatostatin receptor (Polgár, Durrieux, et al., 2013), the aromatase expressing neurons did not. Defining the subtypes of inhibitory interneurons is of particular interest as these subtypes differentially modulate itch and pain (Ross et al., 2010; Duan et al., 2014; Kardon et al., 2014; Bourane et al., 2015; Foster et al., 2015; Petitjean et al., 2015). The sst_{2A}^- inhibitory interneurons, in contrast, are largely uncharacterized. The aromatase-expressing interneurons may, therefore, represent a unique subset of inhibitory interneurons, which could allow for

specific molecular targeting of a nociceptive or pruritoceptive circuit. Future experiments involving ablation or silencing of aromatase neurons and subsequent pain and itch behavioral testing should indicate whether these cells engage modality-specific circuits.

Our finding that approximately 15% of aromatase neurons are activated by capsaicin, formalin, or chloroquine suggests that they indeed contribute to the processing of both pain and itch. Whether individual neurons receive convergent input from nociceptors and pruritoceptors, or whether they are part of hypothesized labeled lines that selectively transmit sensory information (Basbaum et al.; 2009; Bráz et al., 2014), remains to be determined, preferably by electrophysiological analyses. Both capsaicin and formalin have previously been shown to stimulate Fos in inhibitory (GABAergic and/or glycinergic) neurons of the spinal cord dorsal horn (Todd et al., 1994; Hossaini et al., 2010). Conceivably, engagement of theses inhibitory interneurons by algogens and pruritogens underlies a feedforward regulation of output neurons, in a manner comparable to that proposed in Melzack & Wall's Gate Control Theory (1965). Interestingly, Fos (or another marker of activation, phosphorylated extracellular signal-regulated kinase) is only induced in particular subsets of spinal dorsal horn inhibitory interneurons, namely the galanin, neuropeptide Y, and neuronal nitric oxide synthase populations (Polgár, Sardella, et al., 2013). Galanin and neuronal nitric oxide synthase are present in sst_{2A}^{+} cells, whereas neuropeptide Y is in a mix of both sst_{2A}^{+} and sst_{2A}^{-} cells. Because the aromatase-expressing neurons are sst_{2A}^{-}, they may co-express neuropeptide Y. Unfortunately, we could not test this hypothesis as the aromatase reporter mouse produces a nuclear β-gal signal that we could not distinguish from the typical neuropeptide Y punctate staining pattern.

The Fos results also raise important questions about a possible dual estrogenic and inhibitory function of the aromatase-expressing neurons. On one hand, by converting androgens to estrogens via aromatase, these cells may be producing estrogen that increases the excitability of neighboring neurons (Woolley, 2007). On the other hand, because the majority of these cells also express inhibitory markers, they likely release GABA, which would reduce activity of neurons with which they communicate. These seemingly conflicting actions suggest that the aromatase-expressing neurons can concurrently regulate different cell populations by both inhibitory and facilitatory mechanisms. Pharmacological studies that block both estrogen synthesis and inhibitory neurotransmission by these cells could elucidate the functional consequences of a dorsal horn neuron releasing two opposing signaling molecules.

Conclusion

Several groups have suggested that estrogen is synthesized in pain processing areas and contributes to pain. In pursuit of the mechanisms that underlie pain-related local estrogen synthesis, this report represents the first attempt to define the molecular identity of the estrogen-producing cells. We demonstrate that aromatase-expressing cells in the spinal cord and medulla, including the neurons of the nucleus of the solitary tract, are anatomically positioned to receive somatic and visceral nociceptive inputs and have the potential to regulate multiple functions, not only through their capacity to release steroids but also through their neurochemical composition, as they form a distinct subpopulation of inhibitory interneurons. Determining whether the estrogenic and inhibitory components work independently or in concert with each other should generate valuable insights into how these cells exert their influence on nociception.

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2-7. FIGURES

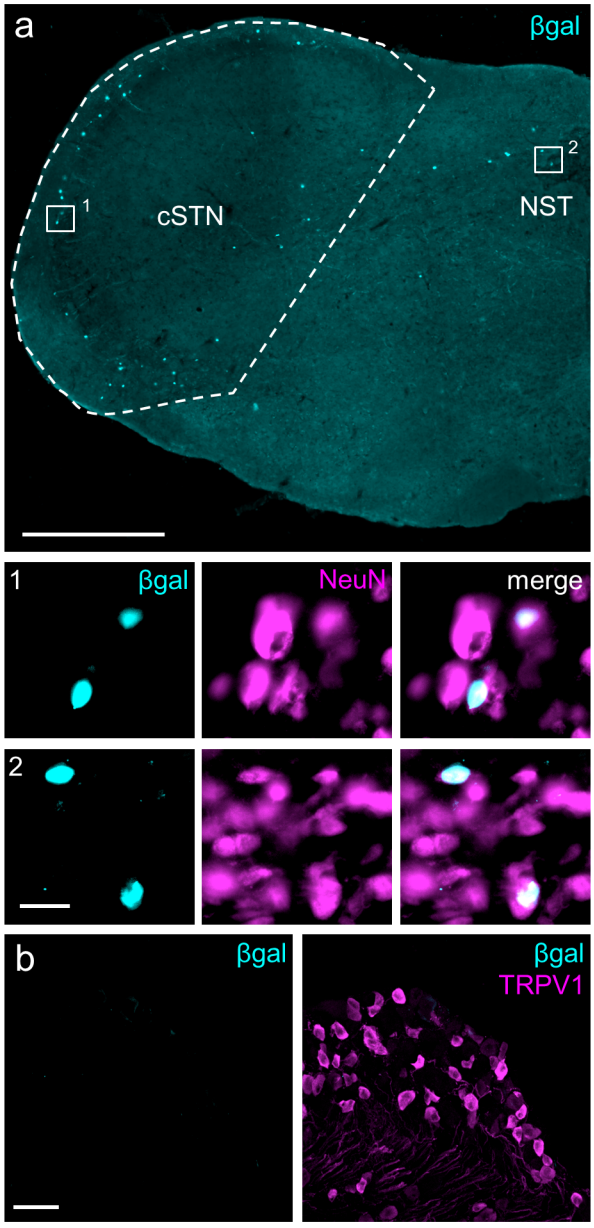


Figure 1

Aromatase expression in the medulla and trigeminal ganglia (TG)

a) Representative section from aromatase reporter mouse illustrates nuclear β-galactosidase (βgal) expression in laminae I and V of the caudal spinal trigeminal nucleus (cSTN, area demarcated by dashed lines) and in the nucleus of the solitary tract (NST, area demarcated by dashed line). Co-staining for the neuronal marker, NeuN, shows complete overlap with β-gal, examples of which can be seen in insets 1 and 2. Image is stitched from single 20X images. Scale bar: 500 μm; insets: 20 μm.

b) No β-gal signal was detected in the TG (left panel). For comparison, right panel illustrates TRPV1-immunoreactive neurons in the same section. Scale bar: 50 μm.

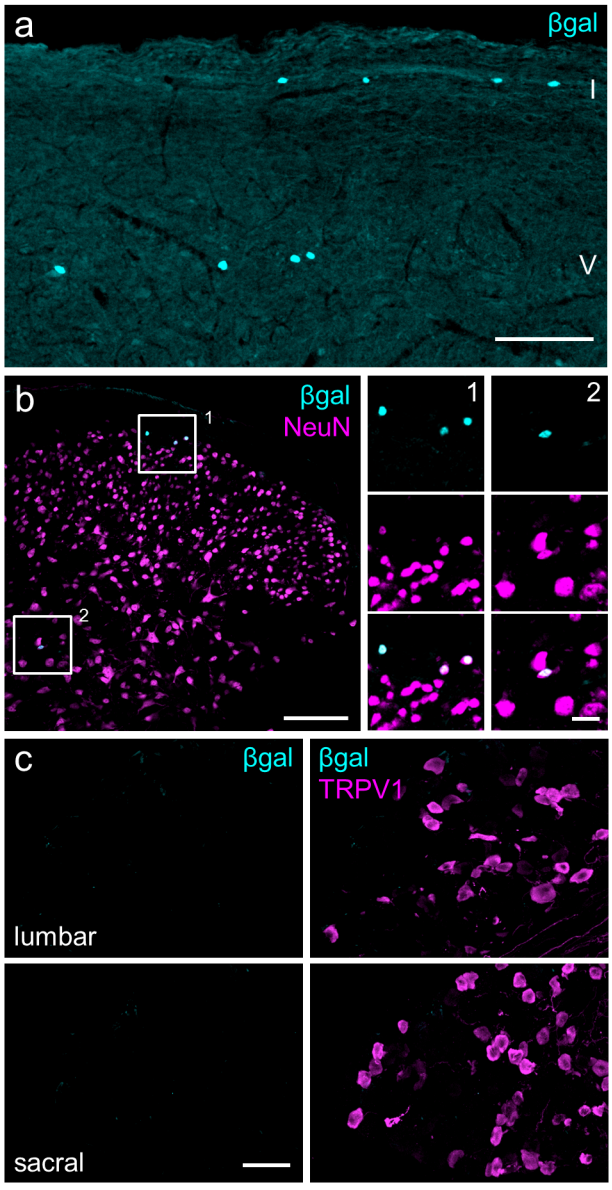

a
βgal
I
V
b
βgal
NeuN
1
2
1
2
c
βgal
βgal
TRPV1
lumbar
sacral

Figure 2

Aromatase expression in the spinal cord and dorsal root ganglia (DRG)

a) Sagittal section of the lumbar spinal cord demonstrates nuclear β-gal expression in laminae I and V of dorsal horn. Scale bar: 100 μm.

b) Coronal spinal cord section immunostained for NeuN. Insets 1 and 2 show co-localization of β-gal with NeuN. Scale bar: 100 μm; inset: 20 μm.

c) Lumbar and sacral DRG do not express β-gal. For comparison, right panels illustrate TRPV1-immunoreactive neurons in the same sections. Images represent maximum Z-projections of confocal images. Scale bar: 50 μm.

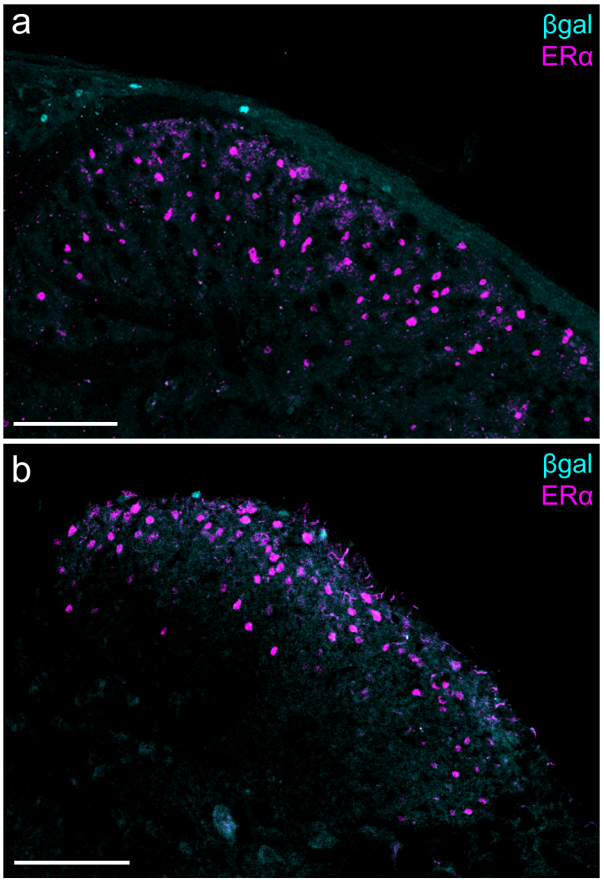

a
βgal
ERα
b
βgal
ERα

Figure 3

Aromatase and estrogen receptor α (ERα) expression in the medullary and spinal dorsal horns

a) Caudal spinal trigeminal nucleus: β-gal^{+} cells are visible in lamina I, whereas ERα-expressing cells are distributed throughout laminae I and II. Scale bar: 100 μm.

b) Sacral spinal cord: There is also no overlap of β-gal and ERα in the spinal cord. Scale bar: 100 μm.

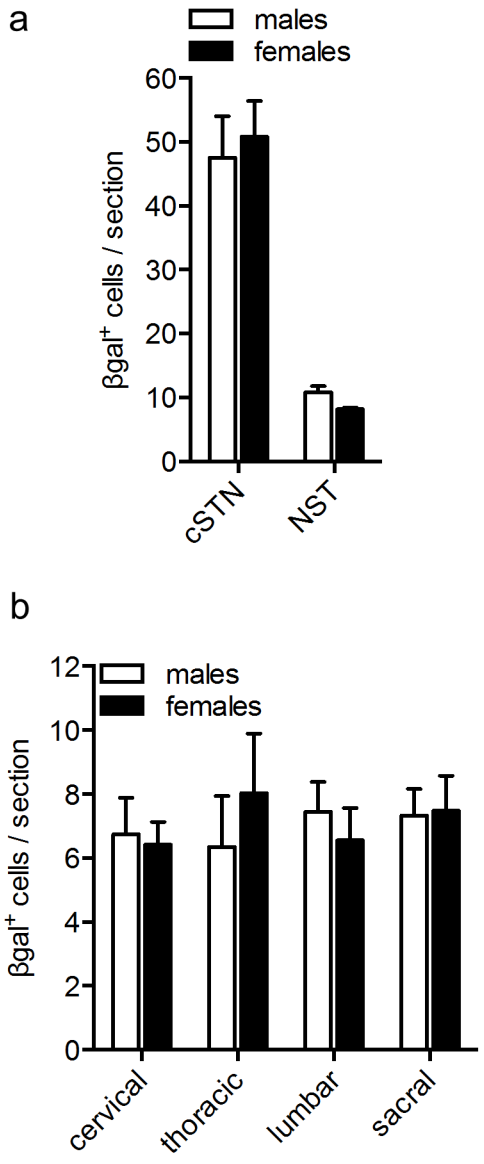

a
males
females
60
50
40
30
20
10
0
βgal+ cells / section
cSTN
NST
b
12
10
8
6
4
2
0
males
females
βgal+ cells / section
cervical
thoracic
lumbar
sacral

Figure 4

No sex differences in spinal or medullary aromatase expression

a) Male and female mice have comparable numbers of β-gal$^+$ cells in the cSTN and NST. Number of β-gal$^+$ cells per section ± SEM — cSTN, males: 54.6 ± 5.9 and females: 56.1 ± 4.8; NST, males: 10.5 ± 0.60 and females: 8.9 ± 0.85. Unpaired *t*-test — cSTN, $p = 0.84$; NST, $p = 0.18$. n = 5 males, 6 females (3-4 sections per animal).

b) Male and female mice also have comparable numbers of β-gal$^+$ cells in cervical, thoracic, lumbar, and sacral spinal cord segments. Number of β-gal$^+$ cells per section ± SEM — cervical, males: 6.7 ± 1.1 and females: 6.4 ± 0.71; thoracic, males: 6.4 ± 1.6 and females: 8.0 ± 1.9; lumbar, males: 7.4 ± 0.94 and females: 6.6 ± 1.1; sacral, males: 7.3 ± 0.83 and females: 7.5 ± 1.1. Two-way ANOVA — effect of sex: $F_{(1,41)} = 0.031$, $p = 0.86$; effect of segment: $F_{(3,41)} = 0.17$, $p = 0.92$; interaction: $F_{(3,41)} = 0.40$, $p = 0.75$. n = 6 males, 5 females (4-6 sections counted per animal at each spinal region).

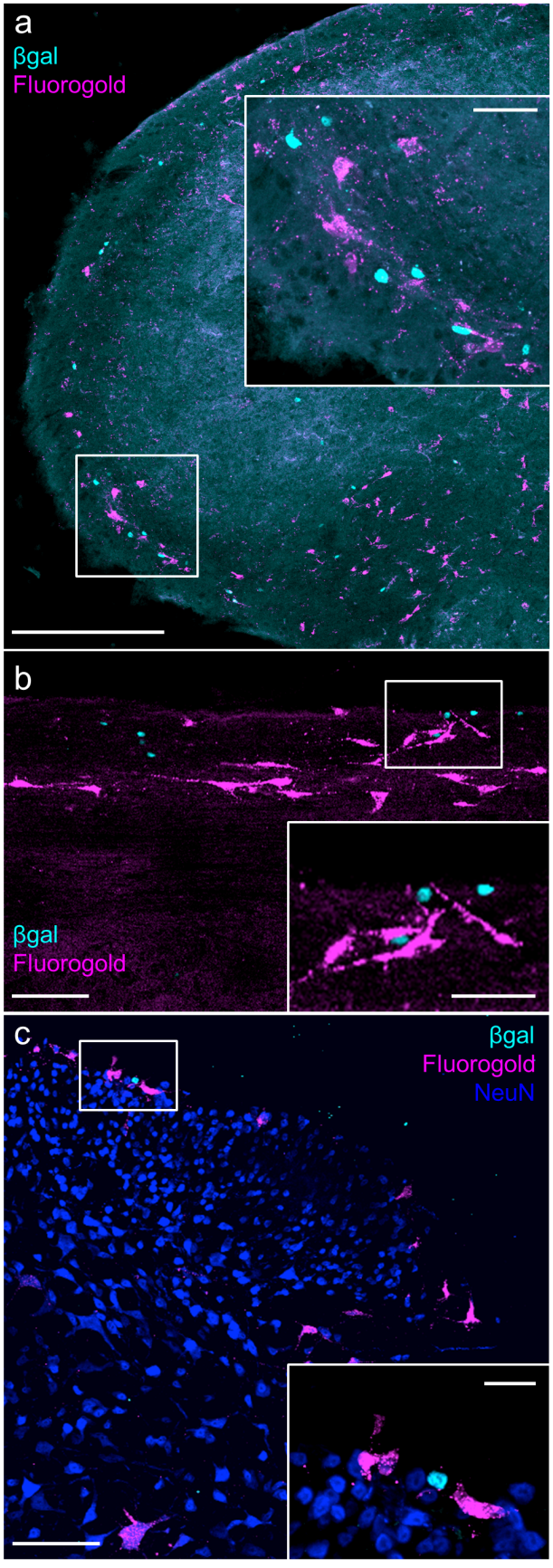
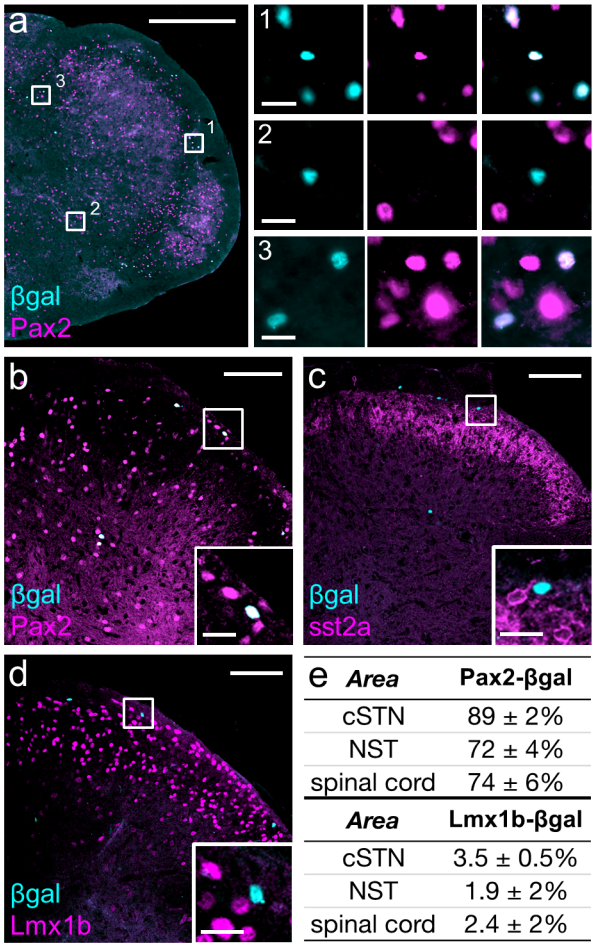

a
βgal
Fluorogold
b
βgal
Fluorogold
c
βgal
Fluorogold
NeuN

Figure 5

Aromatase-expressing neurons do not project to the parabrachial nucleus

a) Representative image of the cSTN 7 days after unilateral injection of a retrograde tracer (Fluoro-Gold) into the lateral parabrachial nucleus. Inset illustrates that β-gal and retrogradely labeled neurons in laminae I and V often appear in neighboring cells, but we never recorded overlap. n = 5 mice (2 males, 3 females). Scale bar: 300 μm; inset: 50 μm.

b, c) Representative images of sagittal (b) and coronal (c) sections of the lumbar spinal cord 8 days after injection of Fluoro-Gold into the lateral parabrachial nucleus. Insets: As for the medulla, we did not record β-gal$^+$ retrogradely labeled neurons. In (c), NeuN immunoreactivity is included to demarcate the most superficial dorsal horn. Scale bar: 100 μm; insets: b) 50 μm, c) 20 μm. n = 6 mice (2 males and 2 females with Fluoro-Gold, 1 male and 1 female with RetroBeads).



e

Area	Pax2-βgal
cSTN	89 ± 2%
NST	72 ± 4%
spinal cord	74 ± 6%

Area	Lmx1b-βgal
cSTN	3.5 ± 0.5%
NST	1.9 ± 2%
spinal cord	2.4 ± 2%

Figure 6

A small subpopulation of medullary and spinal inhibitory interneurons express aromatase

a) Pax2, a marker of inhibitory interneurons, labels the majority of β-gal^{+} cells in the medulla. Insets 1 and 3 show examples of their co-localization in the cSTN and NST, respectively. Inset 2, in the cSTN, illustrates an example in which β-gal and Pax2 do not overlap. Scale bar: 500 μm; inset: 20 μm.

b) Pax2 also co-labels the majority of β-gal^{+} cells in the dorsal horn of the spinal cord. Scale bar (also applies to c, d): 100 μm; inset: 20 μm.

c) β-gal^{+} nuclei were never ringed by cytoplasmic sst2A receptor-immunoreactivity, indicating that aromatase neurons are in the sst2A^{-} subpopulation of inhibitory interneurons.

d) Co-staining for Lmx1b illustrates that very few β-gal^{+} cells are excitatory interneurons.

e) Percentage of β-gal+ cells that co-label with Pax2 or Lmx1b. As an unpaired *t*-test showed no difference between males and females for β-gal and Pax2 or Lmx1b overlap counts from male and female mice were pooled (Pax2: $p = 0.48$, 0.32, and 0.43 for cSTN, NST, and lumbar dorsal horn, respectively; Lmx1b: $p = 0.72$, 0.37, and 0.12 for cSTN, NST, and lumbar dorsal horn, respectively). n = 3 males, 3 females (3-4 sections per animal for the cSTN and NST, 4-6 sections per animal for lumbar dorsal horn).

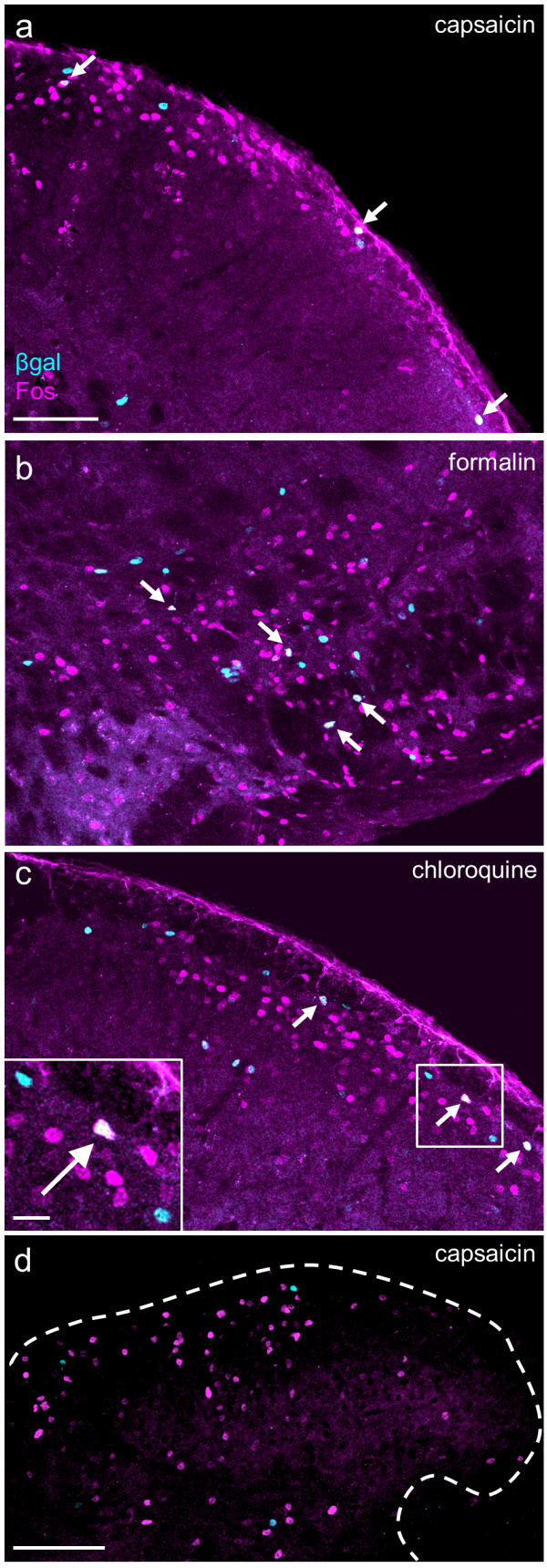

a
capsaicin
βgal
Fos
b
formalin
c
chloroquine
d
capsaicin

Figure 7

Algogens and a pruritogen induce Fos in a small number of aromatase-expressing neurons

a, b, c) Cheek injections of a) capsaicin (5.0 µg / 30 µl; 3 males, 3 females), b) formalin (2% v/v in saline, 50 µl; n = 2 males, 2 females), and c) chloroquine (200 µg in 50 µl; n = 2 males, 2 females) induced Fos expression in a subset of β-gal^{+} neurons in the cSTN (arrows). Scale bar (applies to a, b, and c): 100 µm; inset: 20 µm.

d) In contrast, we did not observe Fos and β-gal overlap in lumbar spinal cord following hindpaw injection of capsaicin (3.0 µg / 10 µl). Comparable results were obtained after hindpaw injection of formalin or chloroquine as well (data not shown). Dashed line outlines border of spinal gray matter. n = 2 males, 2 females. Scale bar: 100 µm.

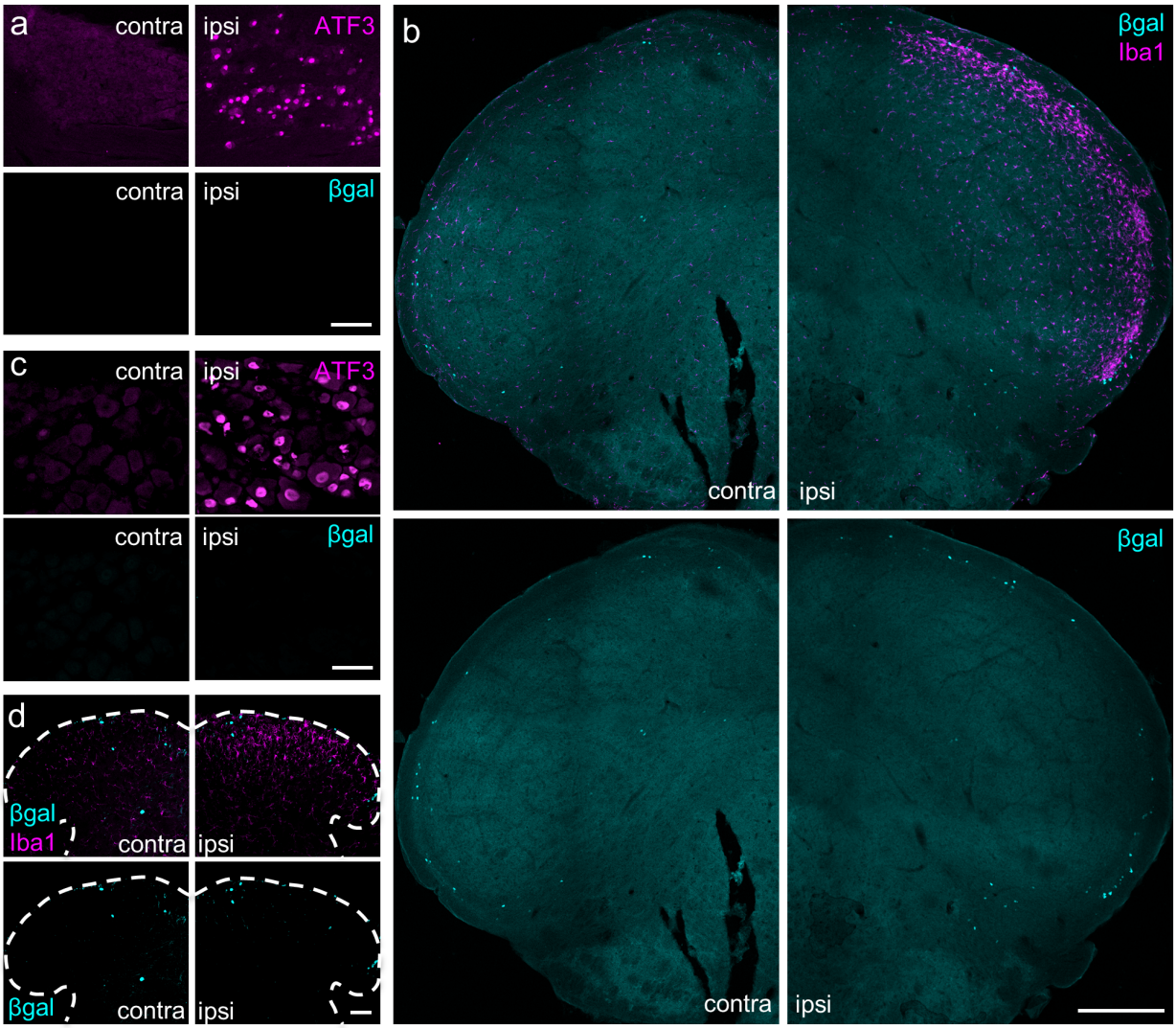

a
contra
ipsi
ATF3
contra
ipsi
βgal
c
contra
ipsi
ATF3
contra
ipsi
βgal
d
βgal
Iba1
contra
ipsi
βgal
contra
ipsi
b
contra
ipsi
βgal
Iba1
contra
ipsi
βgal

Figure 8

Peripheral nerve transection does not alter aromatase expression in primary sensory neurons or in the medullary or spinal dorsal horn

a) Infraorbital nerve transection is a model of facial neuropathic pain. One week after infraorbital nerve transection, which models facial neuropathic pain, axotomized TG sensory neurons express ATF3 (contralateral vs. ipsilateral side, upper panels), but do not upregulate β-gal expression (lower panels). Comparable results were observed in male and female mice. n = 2 males, 2 females. Scale bar: 100 μm.

b) Nerve injury also provoked an upregulation of Iba1+ in microglia ipsilateral to the transection (upper panels), but did not alter the number of β-gal-expressing neurons in the cSTN (lower panels). Comparable results were observed in male and female mice. n = 2 males, 2 females. Scale bar: 300 μm.

c) One week post-injury in a partial sciatic nerve transection model of neuropathic pain, axotomized neurons of the L4/L5 DRG express ATF3 (upper panels), but there is no upregulation of β-gal (lower panels). n = 5 males, 5 females. Scale bar: 50 μm.

d) Similarly, despite significant ipsilateral induction of the Iba1 marker of microglial activation (upper panels), there was no apparent change in the numbers of aromatase expressing neurons (lower panels). n = 1 male, 3 females. Scale bar: 50 μm.

CHAPTER 3

Ablation of dorsal horn ERα-expressing interneurons reduces select modalities of pain and itch

3-1. ABSTRACT

Estrogens are presumed to underlie, at least in part, the greater pain sensitivity and chronic pain prevalence that women experience compared to men. Although previous studies revealed populations of estrogen receptor-expressing neurons in primary afferents and in superficial dorsal horn neurons, there is little to no information as to the contribution of these neurons to the generation of acute and chronic pain. Here we molecularly characterized neurons in the mouse superficial spinal cord dorsal horn that express estrogen receptor α (ERα) and explored the behavioral consequences of their ablation. We found that spinal ERα^{+} neurons are largely excitatory interneurons, many of which co-express substance P, a marker for a discrete subset of nociceptive excitatory interneurons. After viral, caspase-mediated ablation of spinal ERα-expressing cells in male and female mice, we observed a significant decrease in the first phase of the formalin test in males only, and after combining male and female mice, in the second phase of the formalin test. ERα-expressing-neuron ablation also reduced the licking and biting provoked in a chloroquine-induced model of itch, but only in female mice. There were no ablation-related changes in mechanical or heat thresholds, thermal thresholds, capsaicin-induced nocifensive behavior, or histamine-induced pruritofensive behavior. In models of chronic pain, we found no change in Complete Freund's adjuvant-induced thermal or mechanical hypersensitivity, or sciatic nerve injury-induced mechanical allodynia. Finally, in a transneuronal

rabies virus tracing study, we determined that sensory neurons expressing NF200, a marker for myelinated afferents, form the primary input to the dorsl horn ERα^{+} neurons. Thus, ERα labels a subpopulation of excitatory interneurons that are specifically involved in chemically-evoked persistent pain and histamine-independent itch.

3-2. INTRODUCTION

Many chronic pain conditions, such as migraine and temporomandibular joint disorders, are more common in women than they are in men (Unruh, 1996; Berkley, 1997). This sex difference only becomes apparent at puberty and diminishes after menopause (LeResche, 1997; Brandes, 2006). Women are also more sensitive than men on measures of acute pressure, electrical, heat, and cold pain (Fillingim et al., 2009). It is very likely, therefore, that estrogens, the primary female sex hormones, contribute to pain processing.

Estrogens bind to a number of receptors, notably estrogen receptors α and β (ERα and ERβ) and the G-protein-coupled estrogen receptor GPER (Toran-Allerand, 2005; Prossnitz & Barton, 2011). Estrogen receptors are expressed in various organs throughout the body, including the ovaries, testes, liver, lungs, and brain (Couse et al., 1997; Kuiper et al., 1997; Maggiolini & Picard, 2010). As such, they influence functions as diverse as sexual development, immune regulation, and memory (McEwen & Alves, 1999; Gustafsson, 2003). While studies using knockout mice, pharmacology, and gonadectomy and other hormonal manipulations have made valuable contributions to our understanding of estrogenic function in both health and disease (Couse & Korach, 1999; Gustafsson, 2003; Martin, 2009; Paterni et al., 2014), these approaches affect estrogen activity on a global level, making it difficult to establish regional functional specificity of estrogenic contributions (Greenspan et al., 2007; Amandusson & Blomqvist, 2013).

With respect to pain circuitry, estrogen receptors are expressed in both sensory neurons as well as in interneurons of the spinal and medullary dorsal horns (Amandusson et al., 1995;

Williams & Papka, 1996; Papka et al., 1997; Shughrue et al., 1997; Taleghany et al., 1999; Papka et al., 2001; Merchenthaler et al., 2004; Bereiter et al., 2005; Vanderhorst et al., 2005). In addition, in studies of pain, estrogen appears to be pronociceptive in some reports and antinociceptive in others (Kuba & Quinones-Jenab, 2005; Craft, 2007). Whether an interaction of estrogen with these different populations of estrogen receptor-expressing neurons exerts comparable or differential effects is unclear, and may underlie the contradictory conclusions that have been drawn as to estrogen's role in pain processing. A behavioral model of mechanically-induced visceral pain showed that ERα activation in the spinal cord is pronociceptive (Ji et al., 2011), but the effect of spinal ERα in somatic pain or other pain modalities has not yet been reported.

In the present series of studies, we specifically address the contribution of ERα-expressing dorsal horn neurons to nociception. We first determined the extent to which these neurons express markers of excitatory or inhibitory interneurons and next, used a Cre-dependent viral strategy to ablate $ER\alpha^{+}$ cells in the spinal cord dorsal horn of adult mice and evaluated responses to a variety of mechanical, thermal, and chemical stimuli. Several groups (see below) have recently demonstrated that molecularly defined populations of dorsal horn excitatory as well as inhibitory interneurons mediate distinct modalities of pain and itch. At present, we know far more about the function of the inhibitory interneurons than we do of the excitatory interneurons. There are four non-overlapping subsets of inhibitory interneurons and they are distinguished by expression of neuropeptide Y, parvalbumin, galanin/dynorphin, or neuronal nitric oxide synthase (Laing et al., 1994; Polgár et al., 2011; Sardella et al., 2011; Tiong et al., 2011). These populations respond to different modalities of noxious stimuli (Polgár et al., 2013)

and selective loss of these cells in rodents indicates they contribute to mechanical pain (galanin and parvalbumin; Duan et al., 2014; Petitjean et al., 2015), mechanical itch (neuropeptide Y; Bourane et al., 2015), and chemical itch (Kardon et al., 2014). There are also four non-overlapping neurochemical markers for excitatory interneurons: substance P, neurokinin B, neurotensin, and gastrin-releasing peptide (Gutierrez-Mecinas et al., 2016; Gutierrez-Mecinas et al., 2017). With the exception of gastrin-releasing peptide, which is implicated in itch (Sun et al., 2009; Mishra & Hoon, 2013), it is not known what pain and itch modalities, if any, are tightly associated with a particular excitatory interneuron subpopulation. As we describe in this report, the ERα-expressing neurons in the spinal cord dorsal horn are predominantly excitatory interneurons. Their ablation led to a selective reduction in formalin- and chloroquine-induced behaviors, suggesting that the ERα-expressing neurons comprise a functionally distinct subset of excitatory interneurons that mediate chemical pain and itch.

3-3. METHODS

Mouse lines

All experiments were approved by and performed according to the guidelines of the University of California, San Francisco's Institutional Animal Care and Use Committee. For estrogen receptor alpha (ERα) cell ablation experiments, we used ERα-Cre mice, which are mice heterozygous for Cre recombinase that was knocked into the locus of the *Esr1* gene in a manner that preserves expression of ERα (Lee et al., 2014), and their wildtype littermates (ERα-WT).

Viral injections for ablation and knockout

Spinal injection of virus was performed as in a previous publication (Bráz et al., 2012). In brief, mice were anesthetized with ketamine/xylazine (60 mg/kg and 8 mg/kg) and then made a dorsal laminectomy to expose the left side of the lumbar enlargement. Using a micropipette attached to a stereotax-mounted microinjector, we made multiple injections of virus, rostrocaudally along two segments of the lumbar enlargement. Each mouse received a total of 2 µl of viral stock solution, with each injection containing up to 200 nl. For the ERα-Cre cell ablation experiments, we injected AAV1-flex-taCasp3-TEVp (caspase virus, titer: 1.5-2.8x10^{12} viral particles/ml; Gene Therapy Vector Core at the University of North Carolina at Chapel Hill and Dr. R. Jude Samulski; Yang et al., 2013) into ERα-Cre mice and wildtype littermate controls. After injections, mice were allowed to recover from anesthesia. Behavioral testing was begun three weeks after caspase virus injection.

Viral injections for transneuronal tracing and anatomy

Circuit tracing using a rabies virus was performed as previously described (Wall et al., 2010; Bráz et al., 2015). ERα-Cre mice were surgically prepared and dorsal horn injections made as above. However, in these studies, the mice received two different viral injections. The first injection consisted of a rabies helper virus, AAV5-EF1α-FLEX-GTB (TVA-G virus), which expresses the avian TVA receptor and rabies G glycoprotein in infected Cre-expressing cells. Two weeks later, a second injection of the pseudotyped rabies virus, (EnvA)SAD-ΔG-GFP, was done in the same location. This virus only infects cells that contain the TVA-G virus, ensuring that any retrogradely labeled neurons were immediately presynaptic to the spinal ERα-expressing neurons. One week following the rabies virus injection, mice were perfused and processed for immunohistochemistry as described below.

In separate experiments with a Tac1-Cre mouse line (Gutierrez-Mecinas et al., 2017), a Cre-dependent EGFP reporter virus (AAV1-FLEX-eGFP) was injected into the spinal cord dorsal horn to label cells that express substance P. Eight days later, immunostaining was done to evaluate overlap with ERα.

Behavioral tests

For all behavioral testing and scoring, the experimenter was blind to mouse genotype. Post-injection behavioral testing was begun three weeks after caspase virus injection.

Mechanical threshold

Mice were placed into individual acrylic cylinders on a wire mesh and allowed to acclimate for 1-2 hours. Withdrawal responses to von Frey filaments (North Coast Medical, Gilroy, California, USA) applied to the plantar surface of the left hindpaw were recorded and mechanical thresholds were calculated using the up-down method (Chaplan et al., 1994). Mice were tested in a first session prior to virus injection to measure baseline thresholds and again after virus injection to measure post-virus thresholds. For studies examining chronic pain conditions (see below), mice were also tested post-injury. In each session, scores from two consecutive rounds of stimulation were averaged to obtain the mechanical threshold for that session.

Thermal threshold

Mice were placed into individual chambers inside acrylic boxes on a 25.0°C heated glass surface of a thermal nociception test device (Hargreaves et al., 1988; Dirig et al., 1997) and allowed to acclimate for 1-2 hours. Radiant heat intensity was set to 65 units (current output: 4.2-4.5 A) and then the light source was positioned to stimulate the plantar surface of the left hindpaw. Withdrawal latencies to the infrared light were recorded up to a cut-off of 20 seconds. Mice were tested in a first session prior to virus injection to measure baseline thresholds and then in a second session after virus injection to measure post-virus thresholds. For studies examining chronic pain conditions (see below), mice were also tested post-injury. Three readings were taken per session and then averaged to obtain the thermal threshold for that session.

Capsaicin and formalin test

For capsaicin and formalin tests, the mice were placed into individual acrylic cylinders on a glass surface on top of an angled mirror and allowed to acclimate for 30 minutes. Mice were then lightly restrained with a towel and capsaicin (Sigma-Aldrich, St. Louis, Missouri, USA; 3 μg in 10 μl of 10% ethanol, 10% Tween-80, 80% saline) or formalin (10 μl of 2% solution made by diluting 37% formaldehyde 1/50 in saline; ACROS Organics, Morris Plains, New Jersey, USA) was injected into the plantar surface of the left hindpaw with a 100-μl-capacity Hamilton syringe (Hamilton Company, Reno, Nevada, USA) fitted with a 30-gauge needle. Mice were immediately returned to cylinders and video recorded for 5 minutes (capsaicin) or one hour (formalin). Behavior was scored as time spent licking and/or biting the left hindpaw. Formalin behavior was separated into three distinct phases: phase I is defined as the first 0-5 minutes following the injection, interphase is defined as the period 5-10 minutes after injection, and phase II lasted from 10-60 minutes post-injection.

Tests of pruritoception

Using the same cylinders, we made a subcutaneous injection of 100 μl of either chloroquine (200 μg of chloroquine diphosphate salt (Sigma-Aldrich) diluted in saline) or histamine (500 μg of histamine dihydrochloride (Sigma-Aldrich) diluted in saline) into the left calf (LaMotte et al., 2011; Akiyama et al., 2014). Mice were immediately returned to the cylinders and video recorded for 30 minutes. Behavior was scored as time spent licking and/or biting the injection area.

Chronic pain models

Complete Freund's adjuvant (CFA)

To model prolonged inflammation, we injected CFA (Sigma-Aldrich; 20 µl of 1:1 emulsion in saline) into the plantar surface of the left hindpaw of mice lightly restrained with a towel. Three to four days later, when animals display significant paw edema and hypersensitivity (Ma and Woolf, 1996; Malmberg et al., 2003), we used the von Frey and Hargreaves tests to measure mechanical and thermal (heat) thresholds.

Sciatic nerve injury (SNI)

To model neuropathic pain, we performed sciatic nerve injury as described previously (Shields et al., 2003). Under 2% isoflurane anesthesia, we exposed the sciatic nerve and then ligated the peroneal and sural branches. 2 mm of the ligated branches were excised, sparing the tibial branch. The incision was then sutured closed and the mice were allowed to recover and returned to their home cages. One and seven days later, when animals display significant hypersensitivity (Wang et al., 2013; Guan et al., 2016), mechanical thresholds were measured. As SNI does not alter radiant heat sensation (Shields et al., 2003; Wang et al., 2013), we only monitored mechanical sensitivity.

Immunohistochemistry

For marker overlap and viral tracing experiments, we performed fluorescent immunohistochemistry. Mice received an intraperitoneal injection of 250-400 mg/kg Avertin

(2,2,2-Tribromoethanol, Sigma-Aldrich). Failure to respond to a pinch at the base of the tail was used as an indicator of deep anesthesia, at which point the animal was transcardially perfused with 10 ml of phosphate-buffered saline (PBS) followed by 30 ml of 4% formaldehyde (100% formalin) in PBS. Spinal cord and dorsal root ganglia (DRGs) were dissected out and post-fixed in 4% formaldehyde in PBS overnight. Tissue was then cryoprotected in 30% sucrose for at least 12 hours. Spinal cord tissue was cut at a thickness of 25 μm per section on a cryostat, collected in PBS, and then mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA) several hours prior to staining. DRG tissue was cut at 14 μm and directly mounted onto Superfrost Plus slides. For staining, the tissue was blocked for one hour in 10% normal goat serum in PBS containing 0.3% Triton X-100. Primary antibody incubation was done overnight at room temperature. Table 1 provides details of the primary antibodies used. The following day, tissue was washed three times in PBS and then incubated in secondary antibodies for a minimum of two hours at room temperature. Secondary antibodies were Alexa Fluor 488, 594, or 647 raised in goat (Thermo Fisher Scientific, Waltham, Massachusetts) and used at 1:1000 in PBS. Following three final washes with PBS, slides were allowed to dry and then coverslipped using Fluoromount-G aqueous mounting medium (SouthernBiotech, Birmingham, Alabama).

Upon completion of behavioral testing, animals were perfused for immunohistochemistry to quantify numbers of ERα^{+} cells remaining in the lumbar spinal cord dorsal horn. We did not perform fluorescent immunohistochemistry because debris from the injection were auto-fluorescent and were difficult to distinguish from ERα+ cells. Perfusion was performed as above. The lumbar enlargement was subsequently carefully dissected out and then post-fixed and

cryoprotected as above. A notch was cut on the ventral surface of the right spinal cord to mark the side contralateral to the virus injection. On a cryostat, 3 mm of spinal cord was cut in the coronal plane at a thickness of 25-35 μm per section, mounted onto Superfrost Plus slides, and allowed to dry at room temperature for 4-18 hours. Immunohistochemistry was performed following the protocol of Llewellyn-Smith et al., 2005. First, to remove endogenous peroxidase activity, we incubated slides at room temperature in methanol peroxide (1% hydrogen peroxide, 30% methanol, diluted in water) for 30 minutes. The slides were then washed 3 times for 10 minutes each time in 10 mM TRIS base (Trizma, Sigma-Aldrich) and 0.05% merthiolate (Thimerosal, Sigma-Aldrich) in 10 mM phosphate buffer, pH 7.4 (TPBS) that also contained 0.3% Triton X-100 (TPBS + Triton = immunobuffer, IB). Sections were then blocked for a minimum of 30 minutes in 10% normal horse serum (NHS) in IB and ERα primary antibody (rabbit, Millipore, 06-935) incubation took place overnight at room temperature. In these studies, the antibody was diluted 1:20,000 in 10% NHS in IB; this concentration was determined via titration experiments to maximize signal and minimize background staining. The next day, the slides were washed 3 times for 10 minutes each time in TPBS and then incubated overnight at room temperature in biotin-SP-conjugated donkey anti rabbit secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA; diluted 1:500 in 1% NHS in IB). The following day, slides were washed 3 times for 10 minutes each time in TPBS and then incubated for a minimum of 4 hours in ExtrAvidin-Peroxidase (Sigma-Aldrich; diluted 1:1500 in IB). The sections were subsequently washed 3 times for 10 minutes each time in TPBS and then incubated for 10 minutes in a solution of 0.004% ammonium chloride, 0.2% D-glucose, 0.04% nickel ammonium sulfate, and 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride in 10 mM PB, pH 7.4. An equal volume of the same buffer, but containing 2 μl/ml of glucose oxidase was then

added to the slides to yield a final concentration of 1 μl/ml glucose oxidase. After 8 minutes, we stopped the reaction by rapidly rinsing the slides 6-7 times in a row with TPBS, followed by 3-4 rinses with distilled water. After drying at room temperature for several hours, the sections were then cleared by washing twice in xylene and coverslipped using Permaslip mounting medium (Alban Scientific, St. Louis, Missouri, USA).

Table 1. Primary antibodies

Antibody	**Manufacturer**	**Species**	**Concentration**	**RRID**
ERα	Millipore, Cat# 06-935	rabbit	1:10,000-20,000	RRID:AB_310305
NeuN	Millipore, Cat# MAB377	mouse	1:5000	RRID:AB_2298772
PKCγ	Strategic BioSolutions	guinea pig	1:5000	
calbindin	Sigma, Cat #C9848	mouse	1:2000	RRID:AB_2314065
calretinin	Swant, Cat #6B3	mouse	1:5000	RRID:AB_10000320
GFP	Abcam, Cat# ab13970	chicken	1:2000	RRID:AB_300798
GABA	Sigma, Cat# A0310	mouse	1:500	RRID:AB_476667
Lmx1b	T. Müller and C. Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany	guinea pig	1:10,000	RRID:AB_2314752
NF200 (N52)	Sigma, Cat# N0142	mouse	1:10,000	RRID:AB_477257
IB4	Vector Laboratories, Cat# B-1205	biotinylated	1:500	RRID:AB_2314661
CGRP	Peninsula, Cat# T-4032	rabbit	1:1000	RRID:AB_2313775
peripherin	Abcam, Cat# ab99942	rabbit	1:2000	RRID:AB_10863617

Imaging and quantification

Immunofluorescent tissue samples were imaged with ZEN 2010 software (Zeiss) in a LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) using a 20× objective. Images of 3-6 randomly selected spinal cord sections from each mouse were acquired using identical imaging parameters. Images were then processed in Fiji/ImageJ (NIH), which involved cropping, assigning colors to individual channels, brightness and contrast adjustment, maximum intensity projections of Z-stacks, and quantification. For quantification, the Isodata Threshold algorithm was used to define labeled cells in each channel and the Particle Analyzer tool (size range: 15-150 μm, circularity: 0.5-1) was used to count cells. An overlay of the channels was then used to distinguish double-labeled cells. Any changes to brightness and contrast were applied uniformly within a single image and across images from the same experiment.

To count cells in the ablation experiments, the slides were automatically scanned with a 20× objective under brightfield conditions using a Zeiss Axio Scan.Z1 slide scanner. Images were stitched with Zeiss ZEN2 software and then exported to FIJI/ImageJ. The images were converted to 8-bit grayscale and then Brightness/Contrast was modified using the "Auto" feature. Images were then cropped to display only the area from the central canal to the dorsal border of the tissue. To distinguish one side of the spinal cord from the other, we drew a perpendicular line from the central canal to the dorsal border of the cord. Using the Cell Counter tool, an observed blinded to mouse genotype manually counted ERα$^+$ cells on each side of the cord. In our images for quantification, we defined an ERα$^+$ cell as a black circle. To calculate the percentage of ERα$^+$ cells remaining after virus injection, we divided the number of ERα$^+$ cells on the ipsilateral side

by the number of cells on the contralateral side. For our ERα cell ablation experiments, we set a threshold of 25% for ablation; i.e., if an ERα-Cre mouse had less than 25% of ERα cells remaining, we considered it to be a successful ablation and included data generated from this mouse in our analysis, but if more than 25% of cells remained, its data was excluded. Conversely, for ERα-WT control mice, if less than 50% of cells remained, the data were excluded.

Statistical analysis

Statistical analyses were chosen in consultation with the University of California, San Francisco's Clinical and Translational Science Institute. To compare anatomical results between male and female mice, we used Unpaired (Student's) *t*-tests, provided that the data were normally distributed (Shapiro-Wilk test) and demonstrated homogeneity of variances (*F* test). If groups had unequal variances, we used unpaired *t*-tests with Welch's correction. If the groups were not normally distributed, we used the Mann-Whitney *U* test.

For each behavioral test, the results were unblinded and grouped by two factors: sex (male, female) and genotype (ERα-Cre or ERα-fl, ERα-WT). Each of the four resulting groups was then tested for normality using the Shapiro-Wilk test. If data were not normally distributed, all four groups were log transformed to normalize the data so that data would fulfill the requirements for analysis with two-way ANOVA. The following data sets underwent log transformation: ERα cell ablation mechanical threshold, ERα cell ablation capsaicin, ERα cell ablation formalin interphase, ERα cell ablation formalin phase II, and ERα cell ablation

chloroquine. In the case of ERα cell ablation formalin interphase, prior to log transformation, data were translated by adding 1 to all data points because certain datum had a value of 0. All data sets demonstrated homogeneity of group variances as assessed by Levene's test. The Shapiro-Wilk test and Levene's test were performed in Microsoft Excel 2011 using the Real Statistics Resource Pack for Mac (Release 3.5.3), copyright 2013–2017 by Charles Zaiontz, www.real-statistics.com. Data were next transferred to GraphPad Prism (version 6.0h for Mac) for two-way ANOVA with Sidak's multiple comparisons test. We set up two comparisons: 1) ERα-WT males vs. ERα-Cre males and 2) ERα-WT females vs. ERα-Cre females. Statistical significance is as indicated in the figure legends. For non-normal data sets, transformed data were used for statistical analysis but raw data were used in graphs for ease of comprehension. In the experiments where data from male and female were pooled due to low numbers of subjects with successful ablation, we applied *t*-tests or Mann-Whitney *U* tests under the same guidelines described for the anatomical studies.

3-4. RESULTS

Characterization of ERα-expressing cells in the spinal cord dorsal horn

In the spinal cord dorsal horn, ERα-immunoreactive cells are concentrated in superficial laminae (Fig. 1a, left panel) and express the neuronal marker NeuN (Fig. 1a, right panel and insets 1 and 2). Based on the distribution of PKCγ-expressing excitatory interneurons, which mark inner lamina II (lamina II_i; Fig. 1b), it is apparent that the majority of ERα-expressing cells are in outer lamina II (lamina II_o), with some cells in lamina II_i and a few cells in lamina I and in deeper laminae. Furthermore, we found no sex differences in either the number or distribution pattern of spinal $ER\alpha^+$ cells (Fig. 1c).

We previously reported that TR4-Nestin knockout mice exhibit an extensive loss of excitatory interneurons in laminae I and II_o, which results in insensitivity to mechanical stimuli as well as to several algogens and pruritogens (Wang et al., 2013). Figure 2a (left panel) shows that we also detected a huge loss of $ER\alpha^+$ neurons in the spinal cord of TR4-Nestin mice. The few remaining ERα+ cells co-localize with GAD67-GFP, a marker for inhibitory cells (Fig. 2a, middle and right panels). In addition, when we injected the Fluoro-Gold retrograde tracer into the lateral parabrachial nucleus, a region that receives the overwhelming majority of projection neurons from laminae I and V (Hylden, Anton, & Nahin, 1989; Spike, Puskár, Andrew, & Todd, 2003), we never observed Fluoro-Gold labeling in $ER\alpha^+$ cells (data not shown). Taken together, these data suggest that ERα is primarily associated with excitatory interneurons. Moreover, in spinal cord tissue from GAD67-GFP reporter mice (without TR4-Nestin knockout; Tamamaki et

al., 2003), we observed that only 16 ± 5.1% of ERα-expressing cells are also GAD67-GFP$^+$ (n = 1 male, 2 females; Fig. 2b), which further supports that ERα is mainly present in excitatory cells. Calretinin is a marker of a large excitatory subpopulation in lamina II, though it also labels a few cells in lamina I and a small number of inhibitory interneurons (Smith et al., 2015). Co-immunostaining with ERα revealed that 39 ± 2.2% of ERα-expressing cells co-express calretinin (n = 2 males, 1 female; Fig. 2c). We also examined co-labeling for substance P, another marker of dorsal horn excitatory interneurons (Gutierrez-Mecinas et al., 2017). In a substance P reporter mouse (Tac1-Cre injected with Cre-dependent EGFP reporter virus; Gutierrez-Mecinas et al., 2017), we found a high degree of EGFP and ERα co-localization (Fig. 2d). On the other hand, although gastrin-releasing peptide (GRP) also marks a subset of lamina I-II excitatory interneurons (Gutierrez-Mecinas et al., 2016), in a GRP-EGFP reporter line (Mishra & Hoon, 2013), we found no overlap with ERα (Fig. 2e).

Nociceptive and pruriceptive behavioral phenotypes after ERα$^+$ interneuron ablation

To assess whether and to what extent the ERα$^+$ interneurons account for the TR4-Nestin pain and itch insensitivity phenotype, we ablated the ERα-expressing neurons in the dorsal horn by local injection of a Cre-dependent caspase virus (AAV1-flex-taCasp3-TEVp; Yang et al., 2013) in mice carrying the Cre recombinase gene knocked into the ERα locus (ERα-Cre; Lee et al., 2014; Fig. 3a) Their wildtype littermates served as controls (ERα-WT). As expected, we only saw elimination of ERα immunostaining in the dorsal horn ipsilateral to the viral injection in the ERα-Cre mice (Fig. 3b). Importantly, PKCγ immunostaining was preserved in both the ERα-Cre and ERα-WT mice (Fig. 3c), providing more evidence that injection of the caspase virus does not

indiscriminately result in dorsal horn cell death. Although all mice were examined in the behavioral studies, we *a priori* established a minimum degree of cell ablation (<25% of $ER\alpha^+$ cells remained in the ipsilateral spinal dorsal horn compared to the contralateral, uninjected side in an ERα-Cre mouse) to include a mouse in the behavioral analysis. We also considered an ERα-WT mouse with less than 50% of $ER\alpha^+$ cells remaining in the ipsilateral dorsal horn (compared to the contralateral, uninjected side) to have received non-specific damage from the injection and excluded that mouse (Fig. 3d).

Three weeks after viral injection, the mice underwent a battery of nociceptive and pruriceptive behavioral testing (Fig. 4). Full details of the statistical analysis, such as group means and *p* values, are reported in Table 2. In brief, we found statistically significant differences between ablated (ERα-Cre) mice and control (ERα-WT) mice in males for the first phase of the formalin test (Fig. 4d), which is a test of acute chemical pain, and in females, for chloroquine-induced itch (Fig. 4g). However, we should note that in both the first phase of formalin behavior as well as the second phase, which represents persistent chemical pain, sex was not a significant source of variation (Fig. 4f). After pooling results from the male and female mice to compare ERα-WT and ERα-Cre mice, we find that the groups are significantly different (formalin, phase I: two-tailed Mann-Whitney U — $U = 36$, $p = 0.0013$; formalin, phase II: two-tailed Mann-Whitney U — $U = 51$, $p = 0.0087$). In all the above cases, ERα-Cre ablated mice show reduced sensitivity to the stimulus compared to ERα-WT control mice. Independent of the genotype, sex represented a significant source of variation for von Frey mechanical thresholds (Fig. 4a) and formalin interphase (Fig. 4e). Females had higher mechanical sensitivity compared to males and higher responses during interphase, a period associated with active neuronal

inhibition (Henry et al., 1999; Fischer et al., 2014). In contrast, no differences were apparent in Hargreaves thermal (heat) thresholds (Fig. 4b) or capsaicin-induced nocifensive behaviors (Fig. 4c).

Unfortunately, for histamine-induced itch (Fig. 4h) as well as Complete Freund's adjuvant (CFA; Fig. 5a-b) and sciatic nerve injury (SNI; Fig. 5 c-d), models of chronic inflammatory and neuropathic pain, respectively, the numbers of mice with successful ERα^{+} cell ablation were insufficient for analysis by two-way ANOVA. For this reason, we pooled male and female mice with successful ablation numbers and only compared by genotype. In these analyses (Table 2), there were no significant differences between groups in any of the tests or models examined.

Table 2. Nociceptive and pruriceptive behavior in mice after ablation of spinal ERα+ neurons

	ERα-WT male	**ERα-Cre male**	**ERα-WT female**	**ERα-Cre female**	**Two-way ANOVA source of variation**		
Behavioral test	Values presented as mean ± SEM				Sex (male, female)	Genotype (ERα-WT, ERα-Cre)	Interaction
Mechanical (von Frey)	1.13 ± 0.159 g n = 14	1.10 ± 0.108 g n = 14	0.713 ± 0.0823 g n = 11	0.709 ± 0.176 g n = 11	$F_{(1,46)}$ = 8.921 p = 0.0045	$F_{(1,46)}$ = 0.3971 p = 0.5317	$F_{(1,46)}$ = 0.5135 p = 0.4773
			Sidak's multiple comparisons test		WT male vs Cre male: p = 0.9973	WT female vs Cre female: p = 0.6067	
Thermal (Hargreaves)	9.24 ± 1.37 s n = 7	9.43 ± 0.54 s n = 11	9.33 ± 0.74 s n = 7	10.20 ± 0.96 s n = 6	$F_{(1,27)}$ = 0.2279 p = 0.6369	$F_{(1,27)}$ = 0.3479 p = 0.5602	$F_{(1,27)}$ = 0.1449 p = 0.7064

Thermal (Hargreaves), cont.			Sidak's multiple comparisons test		WT male vs Cre male: p = 0.9843	WT female vs Cre female: p = 0.7750	
	ERα-WT male	**ERα-Cre male**	**ERα-WT female**	**ERα-Cre female**	**Two-way ANOVA source of variation**		
Capsaicin	38.12 ± 7.39 s n = 6	25.43 ± 3.63 s n = 12	21.48 ± 2.81 s n = 7	21.54 ± 3.63 s n = 7	$F_{(1,28)}$ = 3.865 p = 0.0593	$F_{(1,28)}$ = 1.723 p = 0.2000	$F_{(1,28)}$ = 1.396 p = 0.2473
			Sidak's multiple comparisons test		WT male vs Cre male: p = 0.1510	WT female vs Cre female: p = 0.9950	
Formalin, phase I	79.55 ± 8.43 s n = 6	42.40 ± 2.98 s n = 11	71.03 ± 8.22 s n = 7	50.17 ± 15.56 s n = 7	$F_{(1,27)}$ = 0.001758 p = 0.9669	$F_{(1,27)}$ = 10.38 p = 0.0033	$F_{(1,27)}$ = 0.8182 p = 0.3737
			Sidak's multiple comparisons test		WT male vs Cre male: p = 0.0116	WT female vs Cre female: p = 0.2286	
Formalin, interphase	1.29 ± 0.54 s n = 6	3.08 ± 0.79 s n = 11	14.83 ± 5.95 s n = 7	7.19 ± 4.67 s n = 7	$F_{(1,27)}$ = 5.226 p = 0.0303	$F_{(1,27)}$ = 0.2286 p = 0.6364	$F_{(1,27)}$ = 4.310 p = 0.0475
			Sidak's multiple comparisons test		WT male vs Cre male: p = 0.4465	WT female vs Cre female: p = 0.1711	
Formalin, phase II	186.5 ± 31.13 s n = 6	128.2 ± 39.37 s n = 11	211.9 ± 36.15 s n = 7	106.7 ± 43.60 s n = 7	$F_{(1,27)}$ = 0.005528 p = 0.9413	$F_{(1,27)}$ = 7.772 p = 0.0096	$F_{(1,27)}$ = 0.04337 p = 0.8366
			Sidak's multiple comparisons test		WT male vs Cre male: p = 0.1386	WT female vs Cre female: p = 0.0948	
Chloroquine	206.9 ± 51.98 s n = 6	167.4 ± 36.47 s n = 12	169.8 ± 36.00 s n = 7	62.35 ± 9.941 s n = 7	$F_{(1,28)}$ = 6.994 p = 0.0133	$F_{(1,28)}$ = 8.985 p = 0.0057	$F_{(1,28)}$ = 2.879 p = 0.1008

Chloroquine, cont.		Sidak's multiple comparisons test	WT male vs Cre male: p = 0.5764	WT female vs Cre female: p = 0.0066	
	WT	**Cre**	**Two-tailed unpaired *t*-test or Mann-Whitney *U* test**		
Histamine	106.4 ± 20.65 s n = 3 males, 4 females	96.06 ± 8.53 s n = 1 male, 3 females	$t_{(9)}$ = 0.3605 p = 0.7268		
CFA, mechanical (von Frey, 3 days)	0.0236 ± 0.0110 g n = 3 males, 3 females	0.0548 ± 0.0363 g n = 1 male, 3 females	$t_{(8)}$ = 0.9811 p = 0.3553		
CFA, thermal (Hargreaves, 3 days)	6.38 ± 1.52 s n = 3 males, 4 females	4.59 ± 0.53 s n = 1 male, 3 females	U = 12 p = 0.7879		
SNI, mechanical (von Frey, 1 day)	0.204 ± 0.0253 g n = 5 males, 3 females	0.119 ± 0.0472 g n = 1 male, 1 female	$t_{(8)}$ = 1.500 p = 0.1719		
SNI, mechanical (von Frey, 7 days)	0.112 ± 0.0181 g n = 5 males, 3 females	0.113 ± 0.0796 g n = 1 male, 1 female	$t_{(8)}$ = 0.01118 p = 0.9914		

Table legend:

Mean ± SEM with number of animals in each group (and sex, if applicable) is reported for each behavioral test. Units of measure for the von Frey data are threshold in grams (g) and the Hargreaves data are withdrawal latency in seconds (s). All other data are reported as duration of licking and biting in seconds (s). Where possible, two-way ANOVA was performed to compare effects of sex (male vs. female), genotype (ERα-WT vs. ERα-Cre; i.e., control vs. ablation), and their interaction. For behavioral tests where the number of subjects was insufficient to analyze across the four groups, data from both sexes was pooled and *t*-test (or Mann-Whitney *U* test if groups were not normally distributed) was performed to compare ERα-WT vs. ERα-Cre (control vs. ablation).

Inputs to spinal ERα-expressing neurons: primary sensory neurons

Having observed rather mixed behavioral phenotypes in the ablated mice, it is difficult to provide a simple circuit explanation for the various deficits. To address this question, we next used a rabies virus-mediated transsynaptic tracing method (Fig. 6a-b) to examine the sources of inputs received by the spinal ERα$^+$ interneurons. In dorsal root ganglia (DRGs), we found that the majority of the cells immediately presynaptic to the ERα-expressing cells (as demonstrated by expression of GFP) are NF200$^+$ neurons (Fig. 6c), which are mainly non-nociceptive Aβ fibers and nociceptive Aδ fibers (Lawson & Waddell, 1991). We found that a small number of peripherin$^+$ and CGRP$^+$ neurons, which correspond to nociceptive unmyelinated C fibers and the peptidergic subset of unmyelinated C and myelinated A fibers (McCarthy & Lawson, 1990; Lawson et al., 1996; Amaya et al., 2000; Seal et al., 2009), respectively, also synapse on dorsal horn ERα$^+$ cells (Fig. 6d-e). We did not find labeling of IB4-binding sensory neurons, a marker for non-peptidergic C fibers (Wang et al., 1994; Dirajlal et al., 2003; Fig. 6f).

3-5. DISCUSSION

In this study, we characterized a subpopulation of excitatory interneurons in the superficial spinal cord dorsal horn that express the estrogen receptor ERα. These neurons co-express the nociceptive neuropeptide substance P and receive inputs from $NF200^+$ primary sensory neurons. After $ER\alpha^+$ neuronal ablation in adult mice, we observe sexually dimorphic deficits in the response to the algogen formalin and to the pruritogen chloroquine. Other nociceptive- and pruriceptive-related behaviors were largely unaffected.

Sex differences mediated by ERα-expressing interneurons

Our finding that spinal $ER\alpha^+$ cells are primarily interneurons in lamina II agrees with previous reports (Amandusson et al., 1995; Williams & Papka, 1996; Vanderhorst et al., 1997). As described by Vanderhorst et al. (2005), we also observed comparable patterns of ERα expression in male and female mice. Quantification of the $ER\alpha^+$ neurons revealed no statistically significant difference between males and females. That there was no difference in number, despite our finding of male-only or female-only behavioral phenotypes after ablation of $ER\alpha^+$ neurons indicates that the sexual dimorphism likely reflects inputs to and outputs of these cells. Thus, it appears that chloroquine and formalin respectively activate a female- and male-specific circuit that involves $ER\alpha^+$ interneurons.

That there are sexual dimorphisms in non-reproductive signaling and behavior is well known—drugs such as opioids have different potencies in men and women and many drugs have

been withdrawn due to an increase in adverse effects in women (Klein et al., 2015). Of course, such observations highlight the importance of performing experiments in both sexes. Unfortunately, due to concerns over fluctuating hormone levels in females during the estrous cycle, most studies are done exclusively in male subjects (Hughes, 2007; Beery & Zucker, 2011). That this may not be critical was emphasized in a meta-analysis of results from male and female mice (Mogil & Chanda, 2005). These authors reasoned that it is not necessary to control for the estrous cycle in female rodents because there are equally relevant fluctuations inmale rodents such as changes in the dominance hierarchy. In fact, the variability in data from females is not larger than the data from males (Prendergast et al., 2014). For these reasons, in the present study we included both male and female mice in all of our experiments without monitoring the stages of estrous. Interestingly, the elevated sensitivity in von Frey mechanical threshold testing and the interphase of the formalin test that we observed in females, as compared to males and independent of the ablation, is consistent with reports by other groups (Gaumond et al., 2002; Mogil et al., 2006), and again stresses the need to test subjects of both sexes.

Behavioral significance of spinal ERα$^+$ neurons

ERα has been previously found to co-localize with markers for the endogenous opioids enkephalin and dynorphin in the dorsal horn (Amandusson et al., 1996; Gintzler et al., 2008) and injection of the algogen formalin induces Fos expression in ERα-expressing cells (Amandusson & Blomqvist, 2010), suggesting that these cells are involved in nociception. We determined that the ERα-expressing neurons are a subset of the population of spinal excitatory interneurons that are eliminated in the TR4-Nestin knockout mouse (Wang et al., 2013). The TR4-Nestin mouse

shows an almost complete loss of the response to von Frey filament mechanical stimulation, capsaicin, formalin, chloroquine, and histamine. This line also displays diminished or no mechanical allodynia following Complete Freund's adjuvant (CFA) or sciatic nerve injury (SNI). In the ERα^{+}-cell-ablated mice, we detected similar defects, but in only two of the behaviors: the formalin test and chloroquine-induced licking and biting. We conclude that ERα-expressing interneurons contribute to a select portion of the TR4-Nestin phenotype, one that is mainly chemical. Other populations of interneurons lost in TR4 mutant mice must underlie the remaining behavioral abnormalities.

The formalin test has two phases of activity and is a model of prolonged pain and inflammation, mimicking post-operative pain. At the dose of formalin we used, 2%, the first phase of formalin-induced nocifensive behavior results from activation of nociceptive as well as normally non-nociceptive primary afferents, whereas the second phase is driven by both primary afferents and sensitized dorsal horn neurons (Taylor et al., 1995; Shields et al., 2010). As NF200^{+} neurons, which are both Aβ and Aδ fibers (Lawson & Waddell, 1991), constitute the bulk of sensory inputs to the ERα-expressing interneurons, it appears that the NF200^{+} afferent population is a necessary contributor to the transmission of formalin-evoked injury. This result is consistent with Puig & Sorkin's (1996) finding that both Aβ and Aδ fibers drive the first phase. As nocifensive behaviors during both phase I and phase II were significantly reduced after ERα^{+} cell ablation, it follows that the ERα-expressing interneurons are major facilitators of formalin-related input from myelinated afferents.

With regard to chloroquine, which binds to the Mas-related G protein-coupled receptor A3 (MrgprA3) and functions independently of histamine (Liu et al., 2009), the circuit mechanisms underlying the behavior we observed are somewhat less clear. Specifically, as MrgprA3 is not expressed by $NF200^+$ sensory neurons (Han et al., 2013), but is expressed by $CGRP^+$ neurons, it is possible that the small number of CGRP-immunoreactive cells we detected in the tracing experiments are sufficient to mediate a behavioral effect. Another possibility is that the ERα-expressing interneurons are downstream of other dorsal horn interneurons that are directly innervated by MrgprA3-expressing afferents. Immunohistochemical characterization of the local cells that are presynaptic to the $ER\alpha^+$ neurons in the dorsal horn would be highly informative in this regard.

ERα marks an excitatory interneuron subpopulation

Neurochemical characterization of dorsal horn excitatory interneurons has demonstrated that there are distinct subtypes, and functional studies have shown that they are differentially engaged in specific modalities of pain and itch. For example, GRP marks a subset of excitatory interneurons that accounts for approximately 11% of all excitatory interneurons in laminae I-II (Gutierrez-Mecinas et al., 2016) and mediates itch but not pain (Sun & Chen, 2007; Mishra & Hoon, 2013). A separate subset, the substance P-expressing interneurons, comprise roughly 20% of laminae I-II excitatory interneurons (Gutierrez-Mecinas et al., 2017). We found that many $ER\alpha^+$ cells, especially those in lamina II_o, co-express substance P, though not all substance P^+ cells are $ER\alpha^+$. As substance P-expressing neurons respond to a variety of algesic and pruritic stimuli (Gutierrez-Mecinas et al., 2017), we may infer that ERα-expressing neurons form a

division within the substance P^+ cells, one that is more specifically responsive to formalin and chloroquine.

Furthermore, as the substance P-expressing neurons are presumed to correspond to the so-called vertical cells (Gutierrez-Mecinas et al., 2017), which have dendrites that are oriented dorsoventrally, they are ideally positioned to receive inputs from myelinated A fibers (Lu & Perl, 2005). Though we do not yet have information as to the dendritic morphology of the ERα-expressing interneurons, it is likely that their ventral dendritic arbor is targeted by the myelinated afferents that we identified in the rabies tracing study. Indeed, myelinated primary sensory neurons that express the vesicular glutamate transporter VGLUT1 terminate in lamina III and deeper in the spinal cord dorsal horn (Brumovsky et al, 2007; Neumann et al., 2008; Kestell et al., 2015), which would be compatible with a circuit wherein the axons of $NF200^+$ primary afferents synapse onto the ventrally extended dendrites of $ER\alpha^+$ interneurons that have their soma in lamina II_o. In ongoing studies, we are using injection of a Cre-dependent fluorescent reporter into ERα-Cre mice so as to visualize both $ER\alpha^+$ cell bodies as well as their dendritic and axonal processes.

Potential for release of peptides by ERα-expressing interneurons

Substance P is a neuropeptide that is released into the dorsal horn by both primary afferent neurons and local dorsal horn neurons (Warden & Young, 1988; Otsuka & Yoshioka, 1993). Genetic deletion of substance P or its receptor, neurokinin 1 (NK1), results in reduced sensitivity to capsaicin, formalin, and higher intensity mechanical and thermal stimuli, as well as

decreased neurogenic inflammation (local inflammation caused by release of inflammatory mediators) without impact on acute mechanical and thermal sensitivity or non-neurogenic (CFA-induced) inflammation and mechanical allodynia (Cao et al., 1998; Felipe et al., 1998). Though the majority of dorsal horn substance P derives from primary sensory neurons (Jessell et al., 1979), we find the behavioral phenotypes observed in the mutant mice are indeed consistent with those in the ERα^{+} neuron-ablated mice (with the exception of capsaicin, where we observed no change). In fact, our data indicate that ERα-expressing interneurons are a substantial source of substance P.

Lamina II_o vertical cells receive monosynaptic excitatory connections from Aδ fibers and make monosynaptic excitatory connections on lamina I neurons (Lu & Perl, 2005). Lamina I contains many projection neurons that express the NK1 receptor, many of which receive noxious input from substance P-expressing primary sensory neurons (Todd et al., 2002). From the above, it could be argued that ERα^{+}, substance P^{+} interneurons also provide a major input to the NK1 receptor-expressing projection neurons, a hypothesis that can be readily investigated by viral tracing experiments in mice that express Cre in NK1 receptor^{+} neurons. Such a circuit raises several intriguing possibilities. As the majority of ERα-expressing interneurons are excitatory, they presumably release glutamate. Given that substance P can potentiate glutamate-induced currents in spinal dorsal horn neurons (Randić et al., 1990), it is conceivable that these signaling molecules are co-released by ERα-expressing interneurons, allowing substance P to enhance the activity of glutamate and thereby strengthen synaptic connections. This synaptic strengthening may contribute to sensitization of dorsal horn neurons as activation of spinal glutamate receptors

and NK1 receptors is linked to the initiation of inflammation-induced hyperalgesia (Malmberg & Yaksh, 1992).

Estrogenic action on spinal ERα$^+$ neurons

Estrogen acting on ERα in the spinal cord is likely to have meaningful implications for pain and itch processing, but we have not yet been able to evaluate this function. As described in Chapter 2, we have identified a population of inhibitory dorsal horn interneurons that express aromatase, the enzyme that catalyzes conversion of androgens (e.g., testosterone) to estrogens. The aromatase-expressing interneurons are concentrated in laminae I and V, placing them in close proximity to the ERα-expressing interneurons, and of course, in regions intimately involved in the processing of pain and itch messages. Estrogen synthesized by the aromatase neurons could diffuse into the ERα-expressing cells, bind the receptor, and activate a variety of downstream signaling pathways (Heldring et al., 2007). In fact, spinally synthesized estrogen has been found to have pro-nociceptive effects, which is consistent with the phenotypes that we observed after ERα$^+$ cell ablation. For example, in male Japanese quails, inhibition of local estrogen synthesis reduced responses to a noxious thermal stimulus (Evrard & Balthazart, 2004), while in male rats, inhibition of synthesis lowered pain scores in the formalin test (Zhang et al., 2012). By selectively knocking out ERα while preserving the neuron, our future studies will more specifically address the contribution of estrogen to the activity of ERα-expressing interneurons.

Conclusion

ERα is expressed by a subset of dorsal horn excitatory interneurons, many of which co-express substance P. As our knowledge of nociceptive and pruriceptive circuitry develops, it has become increasingly clear that molecularly distinct categories of excitatory and inhibitory interneurons in the spinal cord define cell populations that convey different modalities of pain and itch. Functionally, the ERα-expressing interneurons facilitate nociception, notably ongoing pain in the formalin model of postoperative pain, and pruritoception involving the histamine-independent pathway (chloroquine). In addition to their involvement in acute chemonociception, the ERα-expressing interneurons likely co-release substance P and glutamate to modulate the central sensitization that precipitates chronic pain states. Of course, whether and to what extent an estrogenic action upon these ERα-expressing interneurons influences the function of these neurons remains to be determined. Selective deletion of the receptor from these interneurons, without affecting the rather extensive sensory neuron expression of the receptor, should provide answers to those questions.

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3-7. FIGURES

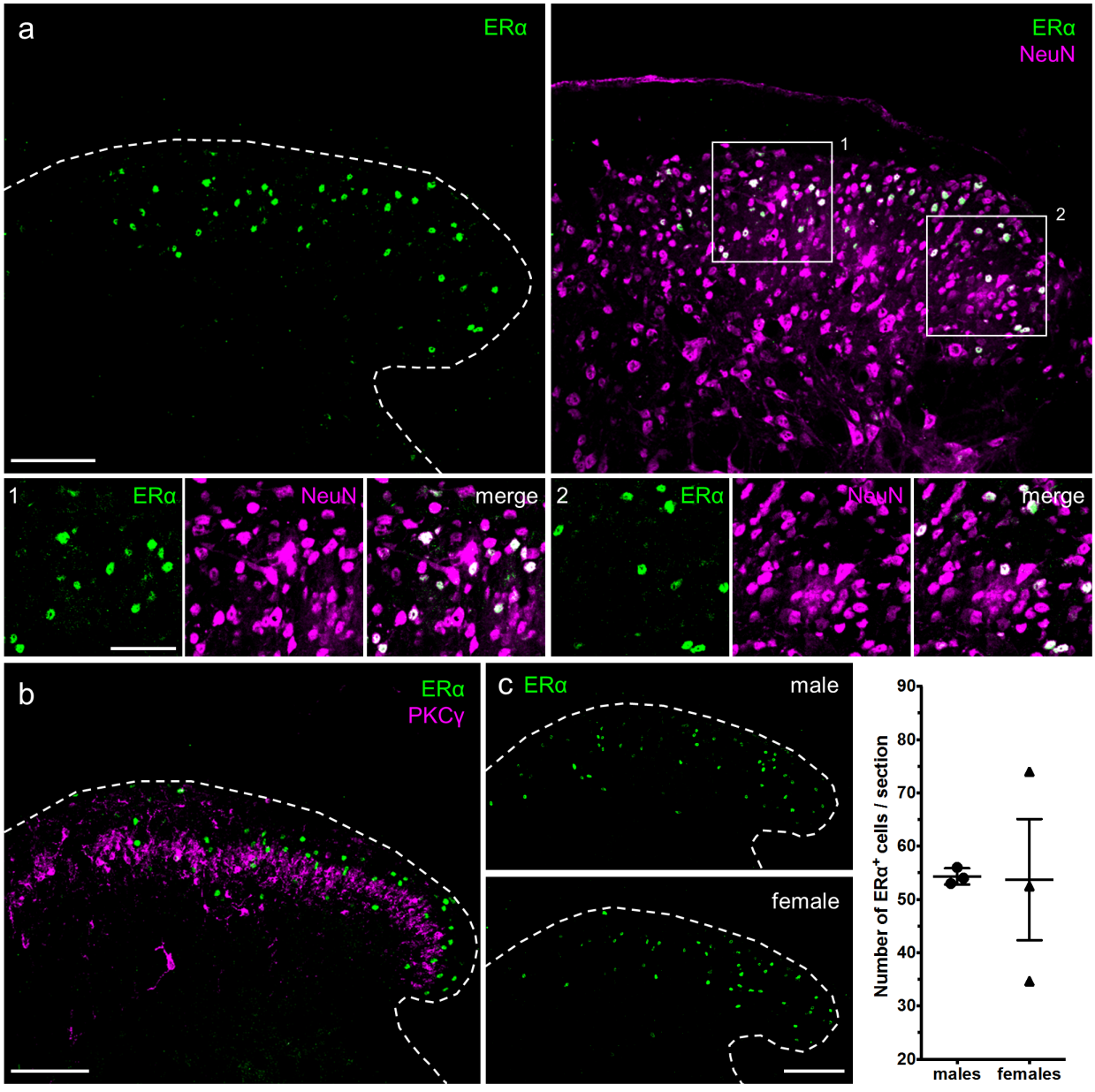
a
ERα
ERα
NeuN
1
2
1
ERα
NeuN
merge
2
ERα
NeuN
merge
b
ERα
PKCγ
c
ERα
male
female
Number of ERα+ cells / section
90
80
70
60
50
40
30
20
males
females

Figure 1

Estrogen receptor α (ERα) expression in the spinal cord

a) ERα is expressed by $NeuN^+$ neurons in the spinal cord dorsal horn. Insets 1 and 2 depict examples of overlap. Dashed line outlines the border of the spinal cord dorsal horn. Scale bar: 100 μm; inset: 50 μm.

b) ERα is mainly expressed by cells of lamina II of the dorsal horn, with scattered cells both superficially and in the deeper laminae (III-V). PKCγ is expressed in a subset of excitatory interneurons and serves as a landmark for inner lamina II. Note that ERα and PKCγ do not overlap. Dashed line outlines the border of the spinal cord dorsal horn. Scale bar: 100 μm.

c) Males and females have comparable numbers of dorsal horn $ER\alpha^+$ cells. Left upper and lower panels display representative images of tissue from a male and female mouse, respectively; right panel displays quantification from 3 males and 3 females. Mean ± SEM (number of $ER\alpha^+$ cells per hemisection) for males: 54 ± 0.88 and females: 54 ± 11. Two-tailed unpaired *t*-test with Welch's correction for unequal variances: $t = 0.05349$, df = 2, $p = 0.9622$. Dashed line outlines the border of the spinal cord dorsal horn. Scale bar (applies to upper and lower panels): 100 μm.

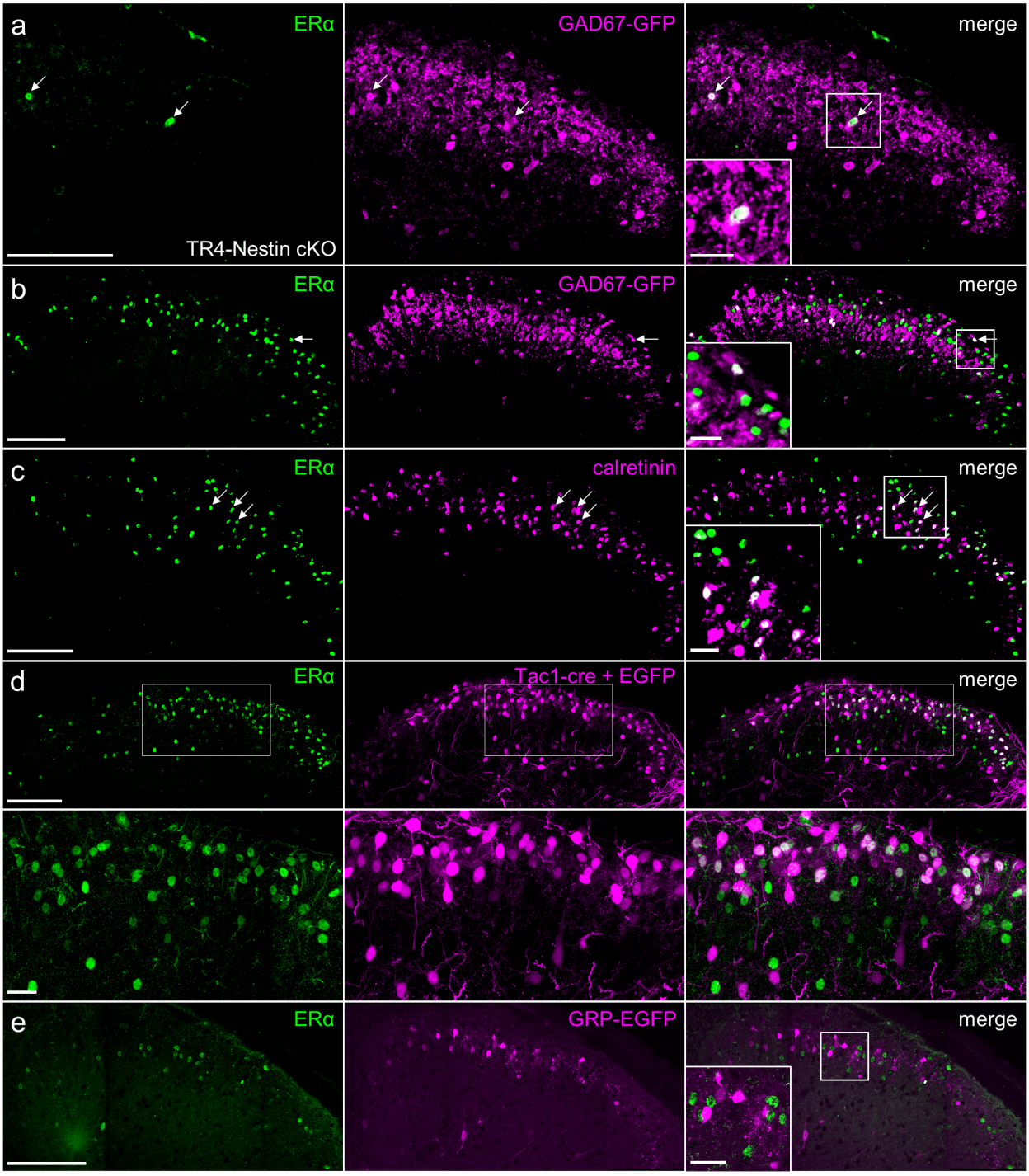
a
ERα
TR4-Nestin cKO
GAD67-GFP
merge
b
ERα
GAD67-GFP
merge
c
ERα
calretinin
merge
d
ERα
Tac1-cre + EGFP
merge
e
ERα
GRP-EGFP
merge

Figure 2

ERα$^+$ neurons are primarily excitatory and co-express substance P

a) A large population of excitatory interneurons in the superficial dorsal horn are missing in TR4-Nestin conditional knockout mice. Staining for ERα in spinal cord tissue from these mice shows that the great majority of ERα$^+$ neurons are lost, indicating that ERα-expressing cells are mainly excitatory. The remaining few ERα$^+$ cells are likely inhibitory, as evidenced by co-localization with GAD67-GFP. Arrows point to examples of ERα$^+$/GAD67-GFP$^+$ cells. Inset illustrates an example of ERα/GAD67 overlap. Scale bar: 100 μm; inset: 20 μm.

b) In tissue from GAD67-GFP reporter mice, only a few ERα$^+$ cells express this inhibitory marker. An example of an ERα$^+$/GAD67$^+$ cell is visible at the arrow and in the inset. Scale bar: 100 μm; inset: 20 μm.

c) Approximately 40% of ERα-expressing neurons immunostain for calretinin, a marker of a subset of excitatory interneurons in lamina II. Arrows depict examples of overlap, which can be seen in detail in the inset. Scale bar: 100 μm; inset: 20 μm.

d) The dorsal horn of Tac1-cre mice were injected with a Cre-dependent EGFP reporter virus to label neurons that express substance P, which is found primarily in excitatory interneurons. This figure illustrates considerable co-expression of ERα and substance P, especially in the most superficial laminae (insets). Scale bar: 100 μm; inset: 20 μm.

e) ERα is not present in gastrin-releasing peptide-expressing neurons, which constitute another subset of excitatory interneurons distinct from the substance P^+ population. Inset shows that GRP-EGFP and ERα immunoreactivity occur in proximate, yet discrete, cells. Scale bar: 100 μm; inset: 20 μm.

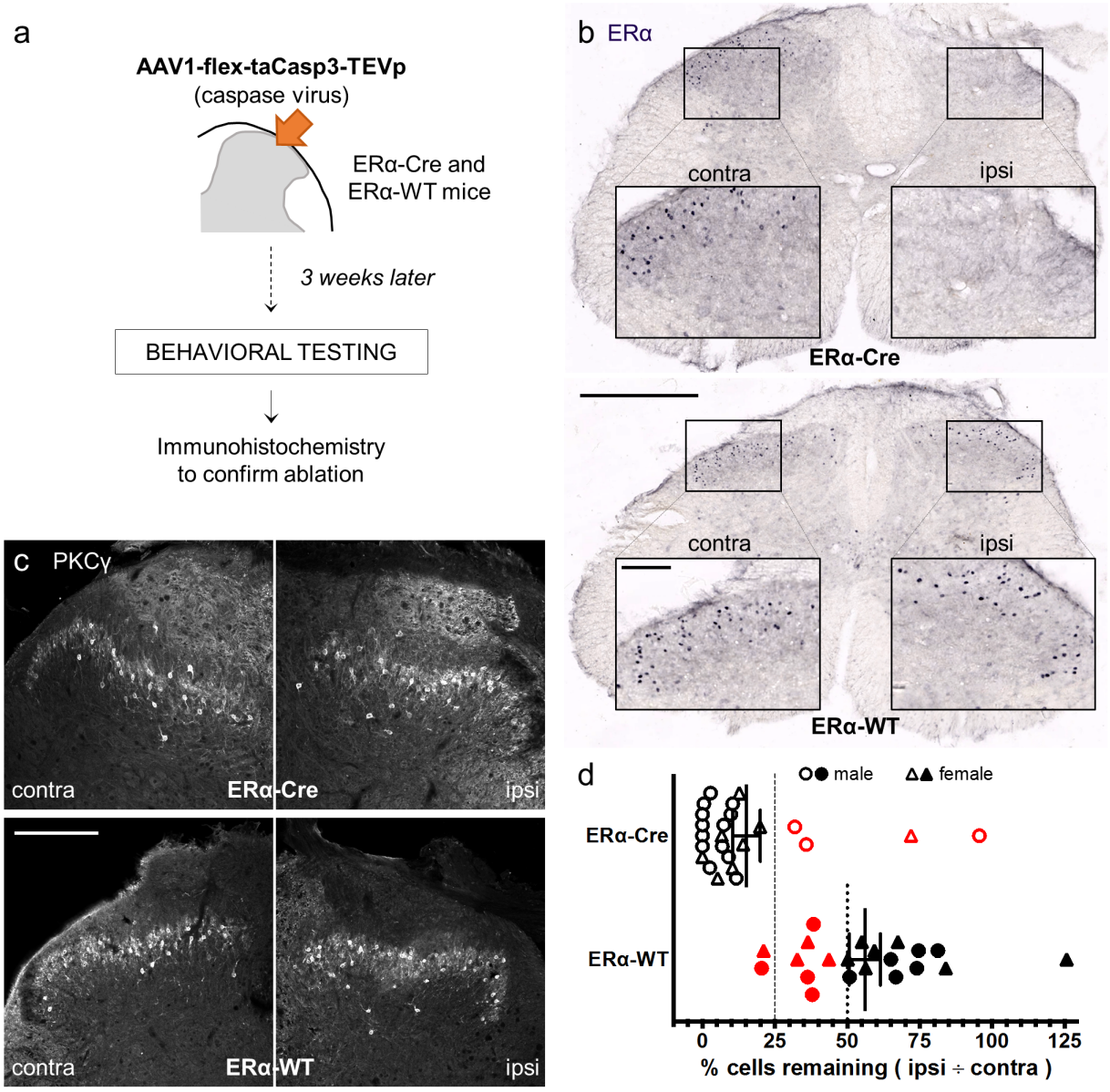
a
AAV1-flex-taCasp3-TEVp
(caspase virus)
ERα-Cre and
ERα-WT mice
3 weeks later
BEHAVIORAL TESTING
Immunohistochemistry
to confirm ablation
b ERα
contra
ipsi
ERα-Cre
contra
ipsi
ERα-WT
c PKCγ
contra
ERα-Cre
ipsi
contra
ERα-WT
ipsi
d
male
female
ERα-Cre
ERα-WT
0
25
50
75
100
125
% cells remaining (ipsi ÷ contra)

Figure 3

Ablation of ERα-expressing cells in the spinal dorsal horn

a) ERα-Cre mice and their wildtype littermates (ERα-WT) received unilateral injections of Cre-dependent caspase virus (AAV1-flex-taCasp3-TEVp) into the lumbar spinal cord dorsal horn. Three weeks later, mice were put through a battery of tests of nociceptive behavior. Upon completion of behavioral testing, spinal cord tissue was immunostained for ERα to assess the quality of the cell ablation in each mouse.

b) Immunostaining for ERα confirms ablation of ERα-expressing neurons in the ERα-Cre mouse (top section) and preservation of the cells in the ERα-WT (bottom section) mouse. The dorsal horn ipsilateral to the viral injection from an ERα-Cre mouse (ipsi, top right inset) exhibits a massive reduction in ERα immunostaining compared to the contralateral side of the same mouse (contra, top left inset) or the ipsilateral side of an ERα-WT mouse (ipsi, bottom right inset). The contralateral side of the ERα-WT mouse (contra, bottom left inset) shows ERα immunoreactivity that is similar to the ipsilateral side. Scale bar: 500 μm; insets: 100 μm.

c) Preservation of the PKCγ staining pattern in the ipsilateral dorsal horn confirms that $ERα^{+}$ cell ablation in ERα-Cre mice does not induce non-specific cell death (top right vs. top left panel). The virus injection in the ERα-WT mice also does not alter PKCγ immunostaining (bottom right vs. bottom left panel). Scale bar (applies to all four panels): 200 μm.

d) Quantification of ERα$^+$ cell ablation illustrates the thresholds that were set to define which mice would be included in subsequent analyses. The percentage of cells remaining following virus injection was calculated by dividing the number of ERα$^+$ cells on the ipsilateral dorsal horn by the number of ERα$^+$ cells on the contralateral side. For ERα-Cre mice, successful ablation was considered to be anything less than 25% (dashed line). For ERα-WT mice, we excluded any animal that had fewer than 50% of ERα$^+$ cells remaining (dotted line). Excluded mice are as indicated in red.

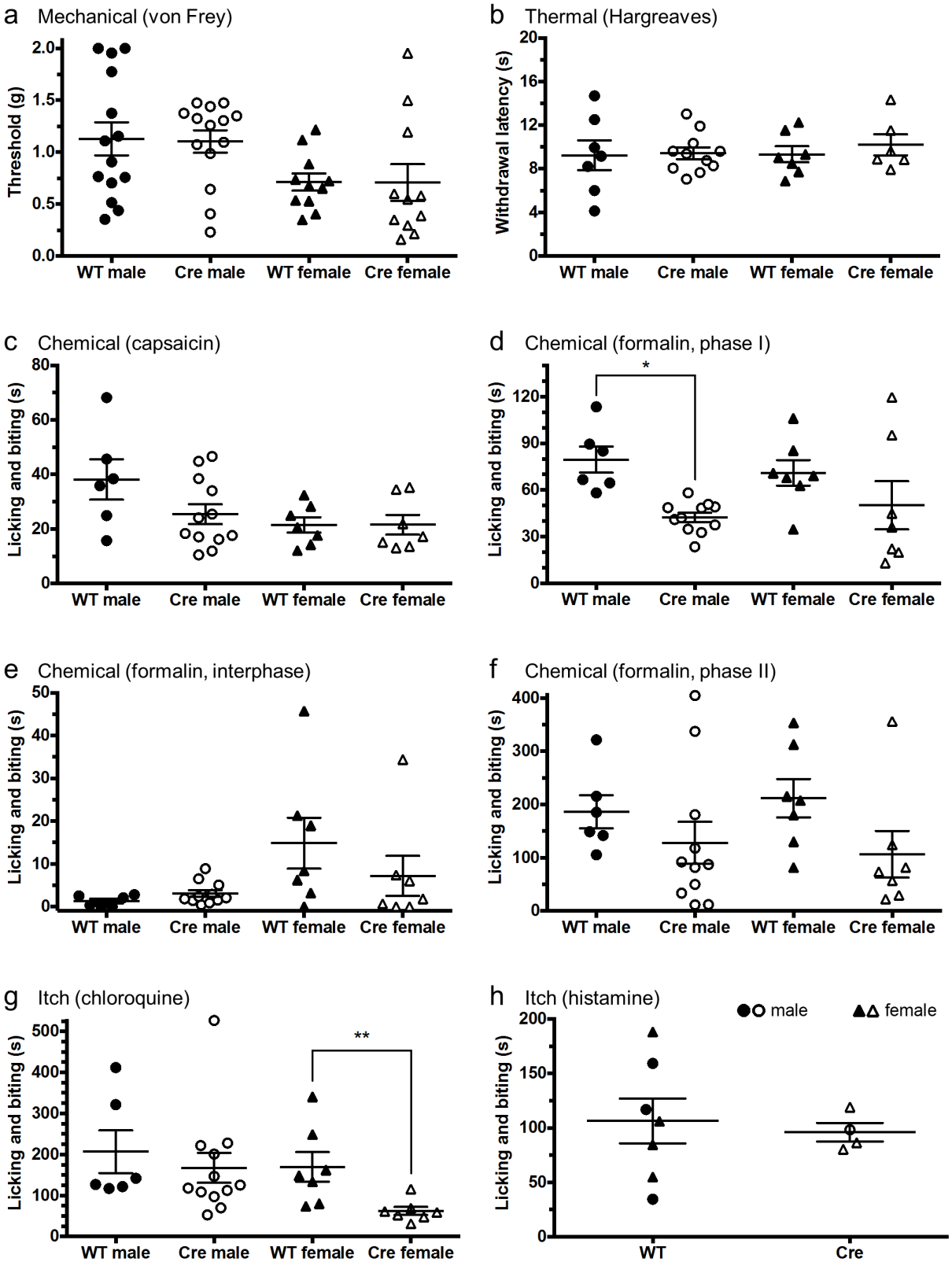
a Mechanical (von Frey)
Threshold (g)
2.0
1.5
1.0
0.5
0.0
WT male
Cre male
WT female
Cre female
b Thermal (Hargreaves)
Withdrawal latency (s)
20
16
12
8
4
0
WT male
Cre male
WT female
Cre female
c Chemical (capsaicin)
Licking and biting (s)
80
60
40
20
0
WT male
Cre male
WT female
Cre female
d Chemical (formalin, phase I)
*
Licking and biting (s)
120
90
60
30
0
WT male
Cre male
WT female
Cre female
e Chemical (formalin, interphase)
Licking and biting (s)
50
40
30
20
10
0
WT male
Cre male
WT female
Cre female
f Chemical (formalin, phase II)
Licking and biting (s)
400
300
200
100
0
WT male
Cre male
WT female
Cre female
g Itch (chloroquine)
**
Licking and biting (s)
500
400
300
200
100
0
WT male
Cre male
WT female
Cre female
h Itch (histamine)
male
female
Licking and biting (s)
200
150
100
50
0
WT
Cre

Figure 4

Acute nociceptive behavior in mice after ablation of spinal ERα^{+} neurons

a) Mechanical thresholds (von Frey test) in the ERα-WT control and ERα-Cre ablated mice did not differ. However, sex accounted for a significant source of variation, with female thresholds lower than male thresholds. Mean ± SEM (threshold in grams) for WT males (n = 14): 1.13 ± 0.159, Cre males (n = 14): 1.10 ± 0.108, WT females (n = 11): 0.713 ± 0.0823, Cre females (n = 11): 0.709 ± 0.176. Two-way ANOVA: interaction of sex and genotype – $F_{(1,46)} = 0.5135$, $p = 0.4773$, sex – $F_{(1,46)} = 8.921$, $p = 0.0045$, genotype – $F_{(1,46)} = 0.3971$, $p = 0.5317$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.9973$ and females – $p = 0.6067$.

b) There is no effect of spinal ERα ablation on the Hargreaves test of heat thresholds. Mean ± SEM (latency in seconds) for WT males (n = 7): 9.24 ± 1.37, Cre males (n = 11): 9.43 ± 0.54, WT females (n = 7): 9.33 ± 0.74, Cre females (n = 6): 10.20 ± 0.96. Two-way ANOVA: interaction of sex and genotype – $F_{(1,27)} = 0.1449$, $p = 0.7064$, sex – $F_{(1,27)} = 0.2279$, $p = 0.6369$, genotype – $F_{(1,27)} = 0.3479$, $p = 0.5602$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.9843$ and females – $p = 0.7750$.

c) Intraplantar capsaicin (3 μg/10 μl)-induced licking and biting of the hindpaw did not differ in ERα-WT control and ERα-Cre ablated mice. Mean ± SEM (duration in seconds) for WT males (n = 6): 38.12 ± 7.39, Cre males (n = 12): 25.43 ± 3.63, WT females (n = 7): 21.48 ± 2.81, Cre females (n = 7): 21.54 ± 3.63. Two-way ANOVA: interaction of sex and genotype – $F_{(1,28)} =$

1.396, $p = 0.2473$, sex – $F_{(1,28)} = 3.865$, $p = 0.0593$, genotype – $F_{(1,28)} = 1.723$, $p = 0.2000$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.1510$ and females – $p = 0.9950$.

d) Nocifensive behavior (licking and biting of the hindpaw) in the first phase of the formalin test (2% formalin, 10 µl, intraplantar), was significantly decreased in male mice after ablation of ERα$^+$ cells (ERα-Cre male) compared to control males (ERα-WT male). Females do not display a significant difference. Mean ± SEM (duration in seconds) for WT males (n = 6): 79.55 ± 8.43, Cre males (n = 11): 42.40 ± 2.98, WT females (n = 7): 71.03 ± 8.22, Cre females (n = 7): 50.17 ± 15.56. Two-way ANOVA: interaction of sex and genotype – $F_{(1,27)} = 0.8182$, $p = 0.3737$, sex – $F_{(1,27)} = 0.001758$, $p = 0.9669$, genotype – $F_{(1,27)} = 10.38$, $p = 0.0033$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.0116$ and females – $p = 0.2286$. * indicates a significant difference between indicated groups at $p < 0.05$.

e) Although we see no significant differences following ERα$^+$ cell ablation, sex and the interaction of sex and genotype form significant sources of variation during interphase in the formalin test. Mean ± SEM (duration in seconds) for WT males (n = 6): 1.29 ± 0.54, Cre males (n = 11): 3.08 ± 0.79, WT females (n = 7): 14.83 ± 5.95, Cre females (n = 7): 7.19 ± 4.67. Two-way ANOVA: interaction of sex and genotype – $F_{(1,27)} = 4.310$, $p = 0.0475$, sex – $F_{(1,27)} = 5.226$, $p = 0.0303$, genotype – $F_{(1,27)} = 0.2286$, $p = 0.6364$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.4465$ and females – $p = 0.1711$.

f) In the second phase of the formalin test, genotype is a significant source of variation, with ablated mice having lower licking and biting times compared to wildtype mice. However, in

multiple comparisons testing, neither of the group comparisons is significantly different. Mean ± SEM (duration in seconds) for WT males (n = 6): 186.5 ± 31.13, Cre males (n = 11): 128.2 ± 39.37, WT females (n = 7): 211.9 ± 36.15, Cre females (n = 7): 106.7 ± 43.60. Two-way ANOVA: interaction of sex and genotype – $F_{(1,27)} = 0.04337$, $p = 0.8366$, sex – $F_{(1,27)} = 0.005528$, $p = 0.9413$, genotype – $F_{(1,27)} = 7.772$, $p = 0.0096$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.1386$ and females – $p = 0.0948$.

g) Licking and biting in response to chloroquine (200 μg/100 μl) injected into the thigh/calf area are significantly decreased in ERα-Cre females compared to ERα-WT females. Spinal ERα ablation does not have a significant effect in males, but sex and genotype are each a significant source of variation. Mean ± SEM (duration in seconds) for WT males (n = 6): 206.9 ± 51.98, Cre males (n = 12): 167.4 ± 36.47, WT females (n = 7): 169.8 ± 36.00, Cre females (n = 7): 62.35 ± 9.941. Two-way ANOVA: interaction of sex and genotype – $F_{(1,28)} = 2.879$, $p = 0.1008$, sex – $F_{(1,28)} = 6.994$, $p = 0.0133$, genotype – $F_{(1,28)} = 8.985$, $p = 0.0057$; Sidak's multiple comparisons test: WT vs Cre, males – $p = 0.5764$ and females – $p = 0.0066$. ** indicates a significant difference between indicated groups at $p < 0.01$.

h) Licking and biting in response to histamine (500 μg/100 μl) injected into the thigh/calf area are not affected by spinal ERα ablation. Male and female data were combined due to the low number of mice. Mean ± SEM (duration in seconds) for WT (n = 3 males, 4 females): 106.4 ± 20.65, Cre (n = 1 male, 3 females): 96.06 ± 8.53. Two-tailed unpaired *t*-test: $t = 0.3605$, df = 9, $p = 0.7268$

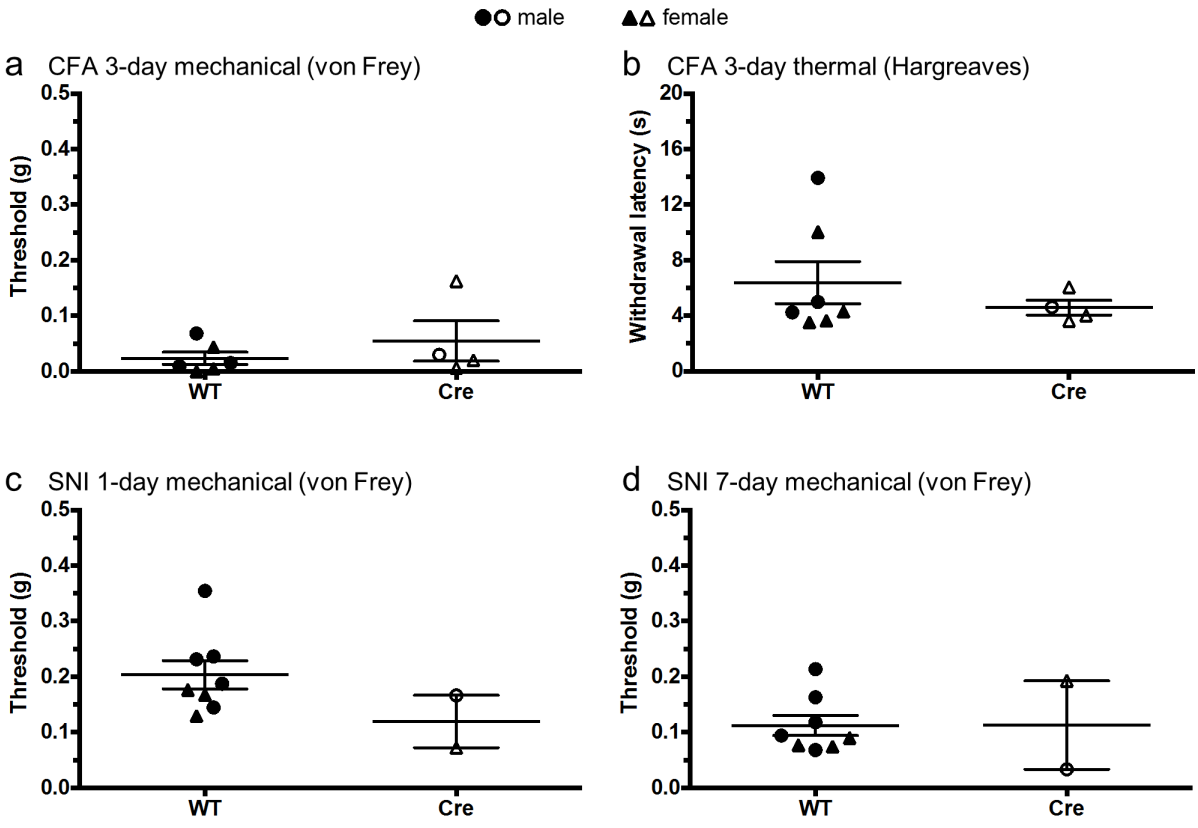
male
female
a CFA 3-day mechanical (von Frey)
Threshold (g)
0.5
0.4
0.3
0.2
0.1
0.0
WT
Cre
b CFA 3-day thermal (Hargreaves)
Withdrawal latency (s)
20
16
12
8
4
0
WT
Cre
c SNI 1-day mechanical (von Frey)
Threshold (g)
0.5
0.4
0.3
0.2
0.1
0.0
WT
Cre
d SNI 7-day mechanical (von Frey)
Threshold (g)
0.5
0.4
0.3
0.2
0.1
0.0
WT
Cre

Figure 5

Models of chronic pain in mice after ablation of spinal ERα^{+} neurons

a, b) Three days after intraplantar injection of Complete Freund's adjuvant (CFA; 20 µl of 1:1 emulsion in saline), thresholds were tested in ERα-Cre mice and WT littermate controls. Males and females were pooled due to the low number of mice. There was no significant difference between groups in neither mechanical allodynia (a) or thermal hyperalgesia (b). Mechanical — mean ± SEM (threshold in grams) for WT (n = 3 males, 3 females): 0.0236 ± 0.0110, Cre (n = 1 male, 3 females): 0.0548 ± 0.0363; two-tailed unpaired *t*-test: $t = 0.9811$, df = 8, $p = 0.3553$. Thermal — mean ± SEM (latency in seconds) for WT (n = 3 males, 4 females): 6.38 ± 1.52, Cre (n = 1 male, 3 females): 4.59 ± 0.53; two-tailed Mann-Whitney *U* test: $U = 12$, $p = 0.7879$.

c, d) One day (c) and seven days (d) after sciatic nerve injury (SNI), mechanical thresholds were tested in ERα-Cre mice and WT littermate controls. Males and females were pooled due to the low number of mice. Note that thermal thresholds do not change in the SNI model and were therefore not tested. There was no significant difference between groups at either time point. 1-day SNI — mean ± SEM (threshold in grams) for WT (n = 5 males, 3 females): 0.204 ± 0.0253, Cre (n = 1 male, 1 female): 0.119 ± 0.0472; two-tailed unpaired *t*-test: $t = 1.500$, df = 8, $p = 0.1719$. 7-day SNI — mean ± SEM (threshold in grams) for WT (n = 5 males, 3 females): 0.112 ± 0.0181, Cre (n = 1 male, 1 female): 0.113 ± 0.0796; two-tailed unpaired *t*-test: $t = 0.01118$, df = 8, $p = 0.9914$.

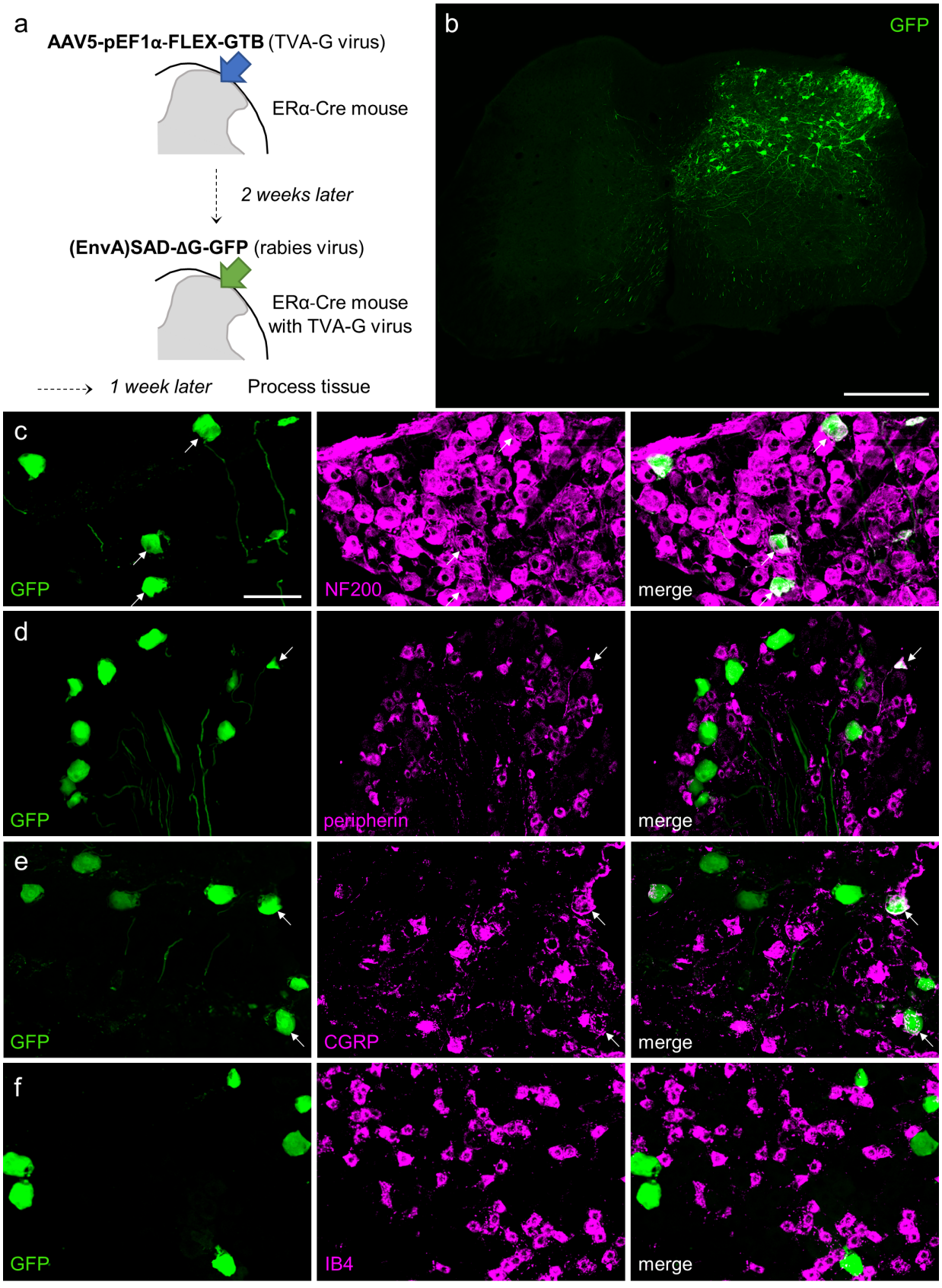
a
AAV5-pEF1α-FLEX-GTB (TVA-G virus)
ERα-Cre mouse
2 weeks later
(EnvA)SAD-ΔG-GFP (rabies virus)
ERα-Cre mouse with TVA-G virus
1 week later
Process tissue
b
GFP
c
GFP
NF200
merge
d
GFP
peripherin
merge
e
GFP
CGRP
merge
f
GFP
IB4
merge

Figure 6

Presynaptic inputs to spinal ERα$^+$ neurons

a, b) Monosynaptic retrograde tracing of immediately presynaptic inputs to spinal ERα$^+$ neurons was carried out by a dual viral injection strategy. In the lumbar dorsal horn of ERα-Cre mice, we first injected a Cre-dependent rabies helper virus containing the avian tumor virus A receptor and glycoprotein (TVA-G virus, AAV5-pEF1α-FLEX-GTB). Two weeks after this injection, in the same location we injected a G-deficient rabies virus pseudotyped with avian envelope A protein that expresses a GFP reporter (rabies virus, (EnvA)SAD-ΔG-GFP). Only cells that contain TVA-G can replicate the rabies virus, restricting the transport of the virus to a single presynaptic neuron. One week after the rabies virus injection, tissue was harvested for immunohistochemistry.

b) An example of rabies infection and transport in the spinal cord dorsal horn can be seen in (b). Although starter and retrogradely labeled cells in the immediate vicinity of the rabies injection cannot be distinguished, there are clear examples of labeled cells in the deep dorsal horn. These are likely sources of presynaptic input to the superficially located ERα-expressing interneurons. Scale bar: 500 μm.

c, d, e, f) Representative images of dorsal root ganglia illustrating sensory neurons presynaptic to the spinal ERα-expressing neurons (labeled by GFP) and immunostained for various primary afferent markers. Arrows shows examples of GFP$^+$ sensory neurons that overlap with the corresponding marker. NF200 (c) marks myelinated afferents (mechanoreceptive Aβ fibers and

nociceptive Aδ fibers). There is very limited labeling of peripherin^{+} neurons (d), which are largely unmyelinated C-nociceptors. Consistent with this, we find almost no retrograde transport to the non-peptidergic population of C-nociceptors, marked by IB4 binding (f). It follows that the co-expression of CGRP (e) in some GFP^{+} neurons represents transport to peptidergic myelinated Aδ nociceptors. Scale bars (c-f): 100 μm.

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