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Neural circuits underlying pain aversion

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This is for you, Mama Lama.

Neural circuits underlying pain aversion

Karuna S. Meda

Abstract

It is commonly hypothesized that hyperexcitability of local circuits within the rostral anterior cingulate cortex (rACC) underlies the affective features of chronic neuropathic pain, but the contribution of specific inputs to the rACC remains unexplored. Here we used optogenetics to study how inputs from the mediodorsal thalamus (MD) and basolateral amygdala (BLA) to the rACC influence pain-related aversion in sciatic nerve injury and chemotherapy-induced mouse models of neuropathic pain. Activating MD inputs elicited conditioned place aversion in mice with chronic neuropathic pain, whereas activating BLA inputs elicited conditioned preference. Strikingly, inhibiting BLA inputs elicited the same effects as activating MD inputs, i.e., exacerbated pain-related aversion. To assess whether these behavioral effects reflect a general pathological dysregulation of rACC neurons vs. changes at specific synapses, we recorded intrinsic properties of layer V rACC pyramidal neurons and their response to light-mediated activation of MD or BLA inputs. We focused on two subtypes of layer V rACC pyramidal projection neurons: intratelencephically-projecting (IT) and subcortically-projecting (SC). In slices from animals with chronic neuropathic pain, we found that both IT and SC neurons are hyperexcitable compared to control. Unexpectedly, however, excitatory responses of these neurons to MD

input activation are significantly weakened in pain conditions compared to control. In fact, in mice with chronic neuropathic pain, the ratio between excitation and inhibition elicited by MD inputs shifted towards inhibition, specifically within SC neurons of the rACC. Responses of IT and SC neurons to BLA input activation are, in contrast, strengthened compared to control. We proposed a model positing that activating MD inputs and inhibiting BLA inputs both drive pain-related aversion through a net inhibition of subcortically-projecting (SC) rACC neurons. Indeed, direct inhibition of SC neurons elicited conditioned place aversion, specifically in mice with chronic neuropathic pain. Inhibition of IT neurons, in contrast, has no effect on pain-related aversion. Our findings reveal a novel role for specific rACC cell types and inputs on pain-related aversion. We conclude that chronic pain-related aversion may be better understood in terms of activity of specific input-output pathways of the rACC, rather than overall activity of the rACC.

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BACKGROUND

Acute and Chronic Pain

Acute pain is a necessary response that alerts us to possible injury or noxious stimuli – a burning flame, a pinprick, or a sprained ankle. However, this pain can sometimes become pathological and persist for months, sometimes longer. This debilitating disorder is chronic pain and affects roughly 25% of the population, with the physical and emotional needs of many patients largely unmet by current therapeutic strategies (Bouhassira et al., 2008; Breivik et al., 2006). Chronic pain can result from tissue injury that is usually associated with inflammation, systemic conditions such as arthritis and cancer, or injury to the nervous system. The hallmarks of chronic pain are allodynia, which is pain that results from normally innocuous stimuli, such as a light touch, and hyperalgesia, which refers to exaggerated pain to already painful stimuli (Basbaum et al., 2009). In conditions of nerve-injury induced pain or neuropathic pain, patients also often experience ongoing or spontaneous pain that occurs in the absence of a physical stimulus. Chronic pain, particularly ongoing neuropathic pain, is often described as shooting, burning and aching and can limit a person's movements, strength, and stamina (Bouhassira et al., 2008; McQuay, 2002). This disorder ultimately impairs the ability to carry out daily-life tasks, leading to disability and severe despair.

The goal of pain management then is to restore a patient's ability to resume these activities and improve general function. Extensive research has uncovered

primary afferent and spinal cord mechanisms of hypersensitivity, and provided multiple bases for pharmacological treatments (Woolf and Salter, 2000; Basbaum et al., 2009; Costigan et al., 2009). Over-the-counter treatments for pain often include drugs like acetaminophen or nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin or ibuprofen. Opioids are often prescribed for strongest pain relief, although there are many undesirable side effects associated with these drugs, including severe constipation and addiction. Some drugs like gabapentin and pregabalin – both anticonvulsants - aim to normalize purported hyperactivity in the nervous system in the setting of chronic pain. However, in many conditions of chronic pain, the pain worsens even though there is no apparent progression of the peripheral processes; central - that is, supraspinal - mechanisms of sensitization are thought to underlie this phenomenon (Melzack and Casey, 1968). Unfortunately, these central mechanisms are not well understood and difficult to target, in large part because drugs that target the central nervous system (CNS) are inevitably associated with more adverse side effects, and surgical procedures are too invasive. Thus the needs of many chronic pain patients are still largely unmet.

Animal Models of Chronic Neuropathic Pain

For our studies, we are interested specifically in chronic neuropathic pain. According to a 2002 study, McQuay found that neuropathic pain is present anywhere from less than 1% (postherpetic neuralgia, trigeminal neuralgia) to almost 30% of the population (neck pain, Reynaud's, chronic lower back pain)

(McQuay, 2002). As mentioned before, neuropathic pain has both an ongoing or spontaneous component, which can be described as shooting, burning, aching and an evoked component (allodynia and hyperalgesia). The pain may be localized (as occurs from hyperactivity of a single peripheral nerve) or widely distributed across multiple dermatomes (generalized hyperactivity of peripheral nerves).

For the purpose of our experiments, we made use of animal models of chronic neuropathic pain. Animal models are critical for studying the mechanisms underlying neuropathic pain and the development of effective therapies for its optimal management. We chose two different models of neuropathic pain: the spared nerve injury (SNI) model consists of a localized injury to the sciatic nerve; specifically two of three branches are cut and ligated (Decosterd and Woolf, 2000). This nerve injury produces a long-lasting increase in the mechanical sensitivity of the affected paw. Specifically, in testing reflexive paw withdrawal to a mechanical stimulus, mice with nerve injury have lower thresholds to normally innocuous mechanical pressure, compared to non-injured animals. As described above, this phenomenon is known as allodynia and is a result of sensitization of sensory neuron and spinal circuits.

We also incorporated a chemotherapy-induced model of neuropathic pain using paclitaxel (Taxol), a common chemotherapy agent used in the clinic. Systemic administration of Taxol leads to widespread neuropathy resulting in whole body

mechanical and thermal allodynia (Polomano et al., 2001; Smith et al., 2004). The ability of pharmaceutical agents to reduce mechanical and/or thermal allodynia in these rodent models for neuropathic pain is often used as a read-out of analgesic or pain-relieving efficacy. However, as is described in more detail below, pain is a multidimensional experience and therefore it is critical to evaluate an analgesic's effect beyond spinally-mediated reflex responses.

Pain is a multidimensional experience

Pain is a subjective experience and multidimensional experience, with sensory-discriminative and affective-motivational aspects. The sensory-discriminative aspect refers to the intensity, location, duration and modality of the painful stimulus. The affective dimension refers to the negative emotional response to a painful stimulus, including unpleasantness, fear or distress and the subsequent urge to escape the unpleasantness (Fernandez and Turk, 1992; Melzack and Casey, 1968). Cognitive processes such as attention to and anticipation or expectation of the pain also affect the magnitude of the pain experience (Villemure and Bushnell, 2009; Wiech et al., 2008). This was illustrated in a study in human subjects who were instructed to rate the unpleasantness of a non-painful (innocuous) warm stimulus. The brain responses and subjective evaluation of the stimulus were compared between two psychologically different contexts: one linked with pain expectation by presenting the non-painful stimuli randomly intermixed with painful stimuli and the other without. Subjects reported higher ratings of unpleasantness when they expected that the stimulus would be

painful (Sawamoto et al., 2000). Expectation of the magnitude or intensity of a painful stimulus can also change the perceived unpleasantness, with higher intensities of expected pain being perceived as more unpleasant (Koyama et al., 1998). The assignment of meaning to pain can also affect its perceived unpleasantness. For example, clinical evidence shows that pain associated with a life-threatening illness like cancer or damaging stimulus like a hot metal rod is perceived as more unpleasant compared to pain that is not associated with any particular damage or consequence (Arntz and Claassens, 2004; Van Damme et al., 2008; Whitehead and Palsson, 1998).

Importantly, the affective component (unpleasantness) can be dissociated from the sensory component (intensity). For example, one study used hypnotic suggestions to either increase or decrease the perceived unpleasantness of a subject's hand immersed in a hot water bath. These suggestions were successful in manipulating the perceived unpleasantness, in either direction. However, these same hypnotic suggestions failed to increase or decrease the perceived intensity of the painful stimulus (Rainville et al., 1999). Importantly, the dissociation of perceived unpleasantness and intensity of a painful stimulus by hypnotic manipulations is accompanied by differential changes in activity of specific brain regions (Rainville 1997) described in more detail below, suggesting that there is an anatomical separation of the processing of these distinct components of the experience of pain.

Indeed, there is strong evidence describing distinct neural pathways that encode sensory-discriminative and affective components of the pain experience (Melzack and Casey, 1968; Melzack and Wall, 1965; Kenshalo and Willis, 1991). The transmission of a painful or nociceptive stimulus begins in the peripheral terminal of a dorsal root ganglion or sensory neuron, which then relays this information to projection neurons in the dorsal horn of spinal cord via its central terminal. From the spinal cord, nociceptive information ascends via distinct pathways. In one pathway - sometimes described as the lateral pain pathway - spinal cord projection neurons send information from the body to the ventral posterolateral (VPL) thalamic nucleus, while input from the face is transmitted by trigeminal pathways to the ventral posteromedial (VPM) nuclei of the thalamus (Willis et al., 1979; Craig et al., 1989). The VPL and VPM act as relays and send information to the primary and secondary somatosensory cortex (S1) and (S2). Neurons in this pathway code for stimulus intensity and duration, and have small, contralateral receptive fields that are somatopically organized (Kenshalo et al., 1988; Chudler et al., 1990; Kenshalo et al., 1980). These properties argue for a sensory-discriminative function.

The spinal cord also sends information to another set of thalamic nuclei, namely the medial and intralaminar thalamic nuclei, in a pathway termed the medial pain pathway (Carstens and Trevino, 1978; Willis et al., 1979; Craig et al., 1989). These thalamic nuclei project to a number of limbic regions, including the amygdala, insular cortex and anterior cingulate cortex (ACC) (Vogt and Sikes,

2000). The medial thalamic nuclei and ACC demonstrate long latency responses to multimodal nociceptive stimuli, with large, and bilateral receptive fields and little somatotopic organization (Dong et al., 1978; Sikes and Vogt, 1992). These properties argue against a sensory-discriminative function and are consistent with the encoding of the affective dimension of pain. Below we take a closer look at the role of the ACC, which is the focus of many of the studies described in subsequent chapters, in encoding pain affect or aversion.

Anterior Cingulate Cortex and Pain Aversion

The anterior cingulate cortex (ACC) encodes the acute aversive response to pain and tonic pain-related affect or aversion in both patients and animal models of chronic pain (Vogt, 2005; Tracey and Mantyh, 2007; Johansen et al., 2001). Positron emission tomography and functional magnetic resonance imaging have demonstrated increases in neural activity in the ACC after noxious stimulation (Casey, 1999) as well as hyperactivation in chronic pain states (Moisset and Bouhassira, 2007; Price, 2000; Treede et al., 1999; Apkarian et al., 2001). Attending to the unpleasantness of a noxious stimulus also increases ACC activity (Rainville et al., 1997), and increased activation of the ACC correlates with both the expectation of pain and the expectation of the unpleasantness of innocuous stimuli (Sawamoto et al., 2000). Furthermore, manipulations to specifically decrease the perceived unpleasantness but not intensity of a painful stimulus are associated with changes in ACC activity, with no effect on activity of the somatosensory cortex (Rainville et al., 1997). A patient with a postcentral

gyrus stroke involving damage mainly to the somatosensory cortex, showed altered pain localization without changes in pain affect (Ploner et al., 1999). In contrast, after cingulotomy, patients reported a decrease in the unpleasantness of their pain, even though the sensory-discriminative features remained intact (Hurt and Ballantine, 1973). The decrease in pain-related unpleasantness even made cingulotomy an early surgical intervention for intractable pain (Folt and White, 1963).

The mechanisms through which the ACC and other limbic structures contribute to the tonic or ongoing aversiveness or unpleasantness that so strongly characterizes the chronic pain phenotype is poorly understood. Studies have shown that there is a functional reorganization of networks involving limbic structures, including the medial prefrontal cortex, (of which the ACC is a part of), insular cortex, and amygdala, across different chronic pain conditions, e.g., chronic back pain, osteoarthritis, and complex regional pain syndrome (Baliki et al., 2006; Baliki et al., 2011; Baliki et al., 2014; Farmer et al., 2012). Another recent study concluded that the transition from acute to chronic pain in humans results from enhanced functional connectivity between the nucleus accumbens and the medial prefrontal cortex (Baliki et al., 2012). These imaging studies, however, have poor spatial and temporal resolution, and invariably lump together numerous cell types, as well as heterogeneous sources of input and diverse outputs. To better understand the mechanisms that contribute to chronic pain-related affect or aversion, it is therefore, critical to examine ACC mechanisms

with a high degree of input and cell-type specificity, and to do so in a way that tests causal relationships on rapid timescales.

Subregions of the Anterior Cingulate Cortex

Before studying the heterogeneity of cells and circuits in the ACC, it is important to note here that there is functional heterogeneity along the rostro-caudal axis of the ACC. In humans, the ACC includes Brodmann Area's 24a/b, 25, 32, and 33. The ACC can be further subdivided based on cytoarchitecture (Vogt et al., 1995a), receptor binding (Palomero-Gallagher et al., 2009), and functional imaging and basal glucose metabolism (Vogt, 2009) into the rostral and posterior ACC. The rostral ACC is further divided into the pregenual and subgenual ACC in relation to genu corporis callosum (corpus callosum). The pregenual ACC has extensive connections with areas involved in processing emotion (amygdala), autonomic functions (lateral hypothalamus and brainstem), memory (hippocampal region), and reward (orbitofrontal cortex, ventral striatum).

In many of the studies described above, the pregenual ACC is specifically implicated in pain-related aversion. In humans, magnetoencephalography demonstrated that the pregenual ACC is associated with delayed activation of nociceptive fibers (via C-fibers) indicating that it is associated with perception of secondary pain, which is characterized by greater unpleasantness and a burning sensation (Ploner et al, 2002). Further, a task in which the location and unpleasantness of noxious cutaneous thermal laser-evoked stimuli were

independently manipulated resulted in significant elevation in pregenual ACC activity when the patient was attending more to unpleasantness (Kulkarni et al., 2005).

The rodent equivalent of Area 24a and b are Cg1 and Cg2, respectively and based on previous studies, we have delineated the rostral ACC using stereotactic coordinates in the mouse (bregma +1.18mm to +2.22mm) (Paxinos and Franklin, 2001). This rostral, agranular region of the ACC receives the most nociceptive input, from the medial and intralaminar thalmic nuclei, including the anteromedial (AM) (Horikawa et al., 1988) and mediodorsal (MD) thalamus, as well as affective input from basal nuclei of the amygdala (BLA) and agranular insular cortex (Mesulam and Mufson, 1982). As will be described in more detail below, this region is also is where the nociceptive neurons reside in the ACC. It also has high expression of delta and mu opioid receptors (Hiller and Fan, 1996; Vogt et al., 1995b; Vogt et al., 1992) and direct injection of morphine into this region is relieves the aversion related to pain in rodents (LaGraize et al., 2006; Navratilova et al., 2015). As will be described below, this rostral portion of the ACC has also been specifically implicated in pain-related aversion in rodent models of chronic pain. For the sake of simplicity, in subsequent chapters, we will simply refer to this region in the mouse as the rACC.

Contribution of rACC to pain-related aversion in animal models

As was described above, many patients with neuropathic pain experience

evoked hypersensitivity that can be mimicked in animal models by testing spinal reflexes. However, many if not most patients with chronic neuropathic pain experience continuous or ongoing pain that is not evoked by an applied physical stimulus, and is likely mediated by supraspinal circuits. Testing reflex withdrawal thresholds in preclinical models is therefore not conducive to uncovering this non-evoked pain or studying the mechanisms underlying it. Previous studies have used the conditioned place preference (CPP) paradigm and the concept of negative reinforcement to uncover the tonic aversiveness or unpleasantness of chronic neuropathic pain in animal models. In this assay, an animal is trained to associate a specific context or environment with a pain state, i.e., pain or no pain, or increased pain or decreased pain. For example, a specific context can be paired with an analgesic or pain-relieving drug. If the animal is experiencing ongoing pain and therefore tonic aversiveness, then it will prefer the analgesic-paired context. If the animal is pain-free, it will show no preference.

King et al (2009) showed that rats with ongoing neuropathic pain developed conditioned place preference to intrathecal clonidine, an analgesic agent used in the clinic. Similarly, blocking descending facilitation via injection of lidocaine into the rostral ventromedial medulla (RVM) region of the brainstem, also induces conditioned place preference in nerve-injured animals. Importantly, when the same experiments are repeated in rats with a lesion of the rACC, those animals with neuropathic pain no longer develop a preference to the analgesic (King et

al., 2009; Qu et al., 2011). Thus, the rACC is critical in encoding the tonic aversive state associated with chronic pain in rodents.

Using the same conditioned place paradigm, Johansen et al (2001) found that the rACC also encodes the acute aversion to painful stimuli. Specifically, excitotoxic lesions of the rACC in rats blocks aversion to an intraplantar injection of the inflammatory agent formalin. Importantly, lesions of the caudal ACC do not affect formalin-induced aversion, indicating a specific implication of the rACC in pain-related aversion in preclinical models as well (Johansen et al., 2001). Similarly, electrolytic lesions of the rACC, lidocaine-mediated inactivation of the cingulum bundle (Vaccarino and Melzack, 1989), and $GABA_AR$ or mu-opioid receptor agonist-mediated inactivation of the rACC (LaGraize et al., 2004; LaGraize et al., 2006; LaGraize and Fuchs, 2007) all reduce avoidance of painful stimuli. Furthermore, nonspecific glutamatergic activation of the rACC produces aversion in the absence of a painful stimulus (Johansen and Fields, 2004). Taken together, these studies support a model in which the rACC processes pain-related aversion in rodents.

Importantly, ablation of the rACC does not affect the latency of acute pain behaviors, such as paw-licking and paw-shaking behaviors produced by intraplantar formalin (Johansen et al., 2001). This finding underscores the dissociation of sensory-discriminative and affective components of pain even in rodents. In controlling for effects on association and memory, Johansen et al

(2001) also demonstrated that rACC lesions do not block conditioned place avoidance (CPA) to other aversive stimuli, for instance a kappa-opioid receptor agonist, which produces dysphoria. Furthermore, rACC lesions in animals with ongoing neuropathic pain did not block preference to inherently rewarding substances, such as cocaine (Qu et al., 2011). Taken together these findings lead to the conclusion that the rACC is specifically associated with pain aversion.

Physiological changes of the rACC in the setting of chronic pain

Neurons that respond to painful stimuli – known as nociceptive neurons – have been identified in the ACC of humans (Hutchison et al., 1999), monkeys (Koyama et al., 1998), rabbits (Sikes and Vogt, 1992) and rats (Yamamura et al., 1996). In contrast to the organization neurons in the somatosensory cortex, these nociceptive neurons are not topographically organized, but rather respond to multimodal noxious stimuli and have large, bilateral or whole-body receptive fields (Hutchison et al., 1999; Yamamura et al., 1996). Nociceptive neurons in the rat are found predominantly in layer V of the rACC and the majority possesses morphological characteristics of pyramidal neurons (Yamamura et al., 1996).

There is also a growing body of research to identify physiological changes in cells and synapses of the rACC in the setting of chronic pain. A series of studies demonstrated that there is increased cellular excitability of layer II/III pyramidal neurons of the rACC (Cao et al., 2009), reduced long-term depression (LTD) and

a concomitant enhancement of long-term potentiation (LTP) of layer II/III synapses following digit amputation or peripheral nerve injury in rats (Wei et al., 1999; Wei and Zhuo, 2001; Xu et al., 2008). In a model of visceral pain, Gong et al (2010) found that the frequency and amplitude of mEPSCs of layer II/III neurons increased, while the amplitude of mIPSCs decreased. This increase in excitatory synaptic transmission in the rACC is presumed to involve both pre-synaptic and post-synaptic mechanisms (Xu et al., 2008). For example, there was enhanced quantal glutamate release in layer II/III pyramidal neurons in models of both chronic inflammatory and neuropathic pain (Toyoda et al., 2009). Post-synaptically, the expression of AMPA receptors in layer II/III is increased in the setting of chronic neuropathic pain and there is increased phosphorylation of specific subunits of AMPA receptors after peripheral nerve injury (Xu et al., 2008). Other studies indicate that kinases involved in long-term potentiation, including Protein Kinase M zeta (PKM ζ) maintain nerve-injury related synaptic potentiation in the rACC (Li et al., 2010).

More recently Blom et al (2014) reported that rACC layer V pyramidal neurons are also hyperexcitable in a nerve injury-induced model of chronic neuropathic pain. These authors also recorded from pairs of layer V pyramidal neurons and inhibitory interneurons and found that there is a specific decrease in the probability of connections of inhibitory interneurons on to pyramidal neurons, i.e., a specific loss in inhibitory synapses. This is indicative of a disinhibition of layer V pyramidal neurons in the setting of chronic pain. Similar to layer II/III, it was also

found that there is an insertion of AMPA receptor subunits in post-synaptic layer V pyramidal neurons following nerve injury (Chen et al., 2014). It is also important to note here that these electrophysiological studies focused on the same stereotactically defined region of the rostral ACC as the behavioral studies described above did.

Cellular heterogeneity of the rACC

The rACC is a highly heterogeneous region, with diverse cell types that target different regions, inhibit or excite their partners, and have distinct physiological and morphological characteristics. Much of this heterogeneity is observed in layer V of the ACC. In the medial prefrontal cortex (mPFC) - of which the rACC is a part - there is no granular layer, which distinguishes it from other cortical areas, notably the somatosensory (S1) and motor (M1) cortex (Van der Werd et al., 2010). As a result, layer V is expanded. For the purpose of our studies, we focused on layer V pyramidal cells because: 1) pain responsive or nociceptive neurons are found in layer V of the rACC (Yamamura et al., 1996), 2) they are the major output neurons of the rACC (Allman et al., 2001), 3) the major afferents of interest, namely the mediodorsal thalamus (MD) and basolateral amygdala (BLA), converge in layer V of the rACC (Musil and Olson, 1988; Sarter and Markowitsch, 1983). This is described in more detail in the next chapters. It is also significant that Blom et al (2014) showed that layer V pyramidal cells are hyperexcitable in a chronic nerve ligation model of neuropathic pain, but whether these effects differed across subpopulations of layer V pyramidal neurons (that

can be differentiated on the basis of their projection targets, morphology, and physiological properties) was not tested.

In fact, several groups have described two distinct layer V pyramidal cell populations in the rACC and neighboring areas of the mPFC (Dembrow et al., 2010; Seong et al., 2012; Gee et al., 2012). One population projects to subcortical structures, including medial thalamic nuclei. These corticothalamic/subcortically-projecting (SC) cells exhibit high levels of hyperpolarization-activated cation current (I_h) that produce a prominent voltage sag and afterdepolarization (ADP) in response to a hyperpolarizing current pulse. The second, corticocallosal/intratelencephalically-projecting (IT) population projects to the contralateral cortex and has a much less pronounced sag or ADP. These differences are illustrated in the next chapter. A more recent analysis of the contribution of HCN channels to pain chronicity did select for layer V rACC neurons that express high levels of I_h (likely SC neurons), and found that peripheral nerve injury induced an activity dependent dysregulation of HCN channels and consequent changes in dendritic integration (Matos et al., 2015; Santello and Nevian, 2015). In a subsequent study by the same group, Santello et al (2017) reported that this dysreguation in HCN channels can be rescued by serotonergic modulation and this rescue is even analgesic in the behaving animal. However, little is known about how cellular heterogeneity contributes to rACC function in encoding pain-related aversion. In the following chapters, we discuss the importance of studying heterogeneous circuits in the rACC and the

extent to which they are differentially recruited in the setting of chronic neuropathic pain.

Inputs to the rACC and their relevance to pain-related aversion

The rACC receives inputs from diverse brain regions, making it well situated as a neural interface between emotion, sensation, and action (Hayden and Platt, 2009). The rACC also shares strong reciprocal connections with regions involved in attention, memory, motivation, emotion, reward, nociception and motor control (Musil and Olson, 1988).

Two major sources of input to the rACC are the mediodorsal thalamus (MD) and the basolateral amygdala (BLA) (Krettek and Price, 1977; McDonald, 1991; Ray and Price, 1992; Hoover and Vertes, 2007). The MD, along with other medial thalamic nuclei, is a major relay for nociceptive information to the rACC (Dong et al., 1978; Hsu et al., 1997). Lesions of the MD, the so-called 'limbic thalamus', block responses of rACC neurons to peripheral noxious stimuli (Hsu et al., 2000; Yang et al., 2006) and severely impair discriminative avoidance learning (Gabriel et al, 1989; Smith et al., 2002). Interactions between the amygdala and the rACC similarly mediate fear learning to painful and other noxious stimuli (Malin and McGaugh, 2006; Bissiere et al, 2008). Lesions of the BLA block avoidance to both intraplantar injection of formalin and a noxious footshock (Gao et al., 2004). Finally, imaging and electrophysiological studies in patients and animal models demonstrated that these regions, like the rACC, are hyperactivate in

chronic pain states (Rinaldi et al, 1991; Mao et al, 1993; Neugeubauer et al, 2004). Taken together, these studies suggest that the rACC combines nociceptive information and contextual information from the MD thalamus and the BLA to guide pain-related behavior.

Using optogenetic tools to dissect rACC circuits involved in pain-related aversion

Many studies describing the functional connectivity between rACC and MD or BLA used pharmacological blockade or lesions. These approaches are limited in that they 1) are inconsistent due to the size and location of lesion sites and inherent complexity and proximity of thalamic and amygdalar nuclei; 2) inevitably destroy axons of passage as well as local neurons and thus 3) do not allow for the study of the contribution of specific cell types, for example excitatory vs. inhibitory neurons and their projections. Studies that used electrical stimulation to activate these pathways in a slice preparation are also limited even in sections preserving thalamocortical or amygdala-cortical pathways, because there is still activation of fibers of passage and axon collaterals, and it is not possible to selectively activate excitatory or inhibitory projections.

The advent of optogenetics has allowed for increased spatial and temporal precision in studying connections and circuits between brain regions. Optogenetics uses a combination of genetic and optical methods to achieve gain or loss of function of well-defined events in specific cells of living tissue (Guru et al., 2015). The technique uses proteins that are highly sensitive to specific

wavelengths of light that can be expressed in specific cell-types and stimulated on rapid timescales (Boyden et al., 2005). Using specific viral serotypes to infect a brain region of choice (for example, MD thalamus), the protein is trafficked to the synaptic terminals of the neurons of interest, to another brain region, where the connection can be manipulated both *in vivo* and *in vitro* by shining light into the target brain region (for example, MD projections to rACC). In our study, we used channelrhodopsin - an opsin that is activated by blue light and allows the influx of cations, thus depolarizing the cell - to activate projections from MD or BLA to the rACC. We also used archaerhodopsin – an opsin activated by green light that pumps protons out of a cell, thus inhibiting it – to inhibit projections from MD or BLA to rACC.

Summary

In the present series of studies, we used optogenetics to explore how inputs from the MD and BLA contribute to rACC-mediated pain aversion. The main questions we ask are:

1) Can MD and/or BLA inputs influence how the rACC encodes pain-related aversion?
2) Are MD and BLA inputs physiologically changed in the setting of chronic pain? How do these inputs recruit output neurons of the rACC?
3) By studying both diverse inputs and output neurons of rACC, can we uncover heterogeneous rACC circuits that differentially contribute to the percept of pain-related aversion?

Chapter 1

The contribution of mediodorsal thalamus inputs to rACC-mediated pain aversion

INTRODUCTION

The mediodorsal thalamus (MD) and its neighboring thalamic nuclei, including the parafascicular, intralaminar, centrolateral nuclei and reuniens, make up the medial thalamic nuclei. These nuclei receive nociceptive input directly from the spinal cord (Giesler et al., 1979; Cliffer et al., 1991), and indirectly from the medullary subnucleus reticularis dorsalis (Villanueva et al., 1991; Villanueva et al., 1998) and the pontine parabrachial nucleus (Bester et al., 1999). They respond to nociceptive stimuli as shown by 1) an increase in cFos expression in the medial thalamic nuclei observed in rats which have undergone a noxious colorectal distension (Traub et al., 1996) or electrical stimulation of hind limb sensory nerve C-fibers (Pearse et al., 2001) 2) short-latency responses recorded by extracellular recordings to electrical stimulation of the sciatic nerve and mechanical stimulation of the hindpaw. In concert, the medial thalamic nuclei make up the primary source of nociceptive information to the rACC (Dong et al., 1979; Hsu et al., 2000; Yang et al., 2006). Importantly, lidocaine injections into or lesions of medial thalamic nuclei block nociceptive responses of rACC neurons (Hsu et al., 1997, Hsu et al., 2000). Medial thalamic nuclei, like the rACC, demonstrate long latency responses to nociceptive stimuli, with large, bilateral receptive fields and little somatotopic organization (Dong et al., 1978, Sikes and

Vogt, 1992). These properties are consistent with the MD and other medial thalamic nuclei encoding features of pain affect and transmitting them to the rACC. Furthermore, imaging studies in humans demonstrated an increase in spontaneous discharge of medial and intralaminar nuclei in patients with deafferentation pain (Rinaldi et al., 1991). Autoradiographic techniques to measure glucose metabolism found similar increases in activity of medial thalamic nuclei in a rat model of peripheral neuropathy (Mao et al., 1993). Finally, extracellular multi-unit recording in rats demonstrated increase in spontaneous firing rate of MD in the setting of spinal cord injury (Whitt et al., 2013).

The MD is also heavily connected with limbic brain areas, including the medial prefrontal cortex (mPFC) and insular cortex, striatum and the amygdala (Krettek and Price, 1977; Ray and Price, 1992; Velayos et al., 1985). Indeed, the connection between MD and mPFC (including the rACC) has been implicated in a variety of cognitive functions, including working memory, behavioral flexibility and avoidance learning (Parnadeau et al., 2017). The electrophysiological connection between the MD and the rACC/mPFC has been characterized primarily through extracellular recordings and slice physiology. These studies have described predominantly excitatory responses of rACC neurons following MD stimulation that can be grouped into short-latency and long-latency responses. The short-latency responses results from the orthodromic activation of MD-rACC pathway, while the long-latency responses results from activation of recurrent collaterals of antidromically driven corticothalamic fibers (Pirot et al.,

1994). These limitations of electrical stimulation however have not allowed for more specific characterization of MD-rACC synapses. Moreover, while MD-rACC connection has shown to be critical for transmission of acute nociceptive or painful stimuli, the physiological nature of this connection has not been studied in the setting of chronic neuropathic pain.

The MD therefore presents itself as a unique target because it is positioned in nociceptive, cognitive, and affective processing streams. Our study provides novel evidence for the ability of MD inputs to influence representations of pain-related aversion in the rACC.

RESULTS

MD inputs to the rACC exacerbate pain-related aversion

We first addressed the effect of MD input activation on pain-related aversion using a conditioned place preference (CPP) paradigm (**Fig. 1A**) that measures ongoing aversiveness associated with chronic neuropathic pain (Qu et al., 2011). To activate excitatory inputs to the ACC, we injected an adeno-associated virus (AAV5) that drives expression of a channelrhodopsin-2 (ChR2)-yellow fluorescent protein (eYFP) fusion protein under control of the CaMKIIα promoter into the MD and then implanted an optical fiber over the rACC (**Fig. 1B**). For these experiments, we modeled the chronic neuropathic pain condition by performing spared nerve injury (SNI) or injecting Taxol systemically, 3 weeks after viral injection and optical fiber implantation. CPP was performed 7-10 days after the SNI procedure or Taxol injections, allowing for a total of 4-5 weeks of viral expression (**Fig. 1B**). In this assay, the effect of the conditioning stimulus can be quantified using the preference score or “Preference for the Stim Side” (namely the ratio of time spent in the stimulated side after vs. before conditioning) and/or the ‘Difference Score’ (namely the difference of time spent in a chamber post vs. pre conditioning). Stimulation that exacerbates pain-related aversion should elicit conditioned aversion in animals with chronic pain, but not in pain-free controls, resulting in a Preference Score (for the stimulated side) <1. Conversely, stimulation associated with attenuation of pain-related aversion should elicit conditioned preference in animals with chronic pain (indicated by a Preference Score >1). After conditioning (see **Methods**), we found that animals

with chronic neuropathic pain (either SNI or Taxol) avoided the chamber paired with optogenetic activation of MD inputs to rACC (Preference Score significantly <1). In contrast, Control animals neither preferred nor avoided the side paired with activation of MD inputs (**Fig. 1C**). This result was also reflected by corresponding changes in the Difference Scores (**Fig. 1–figure supplement A** and **B**). Thus, activation of MD inputs to the rACC exacerbates pain-related aversion.

Even with optogenetic stimulation, there is the risk of antidromic activation. Specifically, optogenetically activating MD terminals could elicit back-propagating action potentials that might activate MD neurons targeted by the rACC. We therefore used optogenetic inhibition to confirm that the behavioral effects of MD terminal activation reflect local effects of MD inputs within the rACC. Here we carried out experiments using the same timeline as before, but now we inhibited MD inputs to rACC using archaerhodopsin (Arch) driven by a Synapsin promoter (AAV5-hSyn-eArch-eYFP) (**Fig. 1D**). Indeed, Arch-mediated inhibition of MD inputs elicited effects opposite to those of activating MD inputs, i.e., animals with chronic neuropathic pain developed a preference for the chamber paired with optogenetic inhibition (preference score significantly >1). Interestingly, we observed a conditioned preference for the chamber paired with optogenetic inhibition of MD inputs in control animals as well (**Fig. 1E.** Difference scores represented in **Fig. 1–figure supplement C**). Thus, inhibition of MD inputs to the rACC reveals that these inputs transmit an aversive signal. Optogenetic

activation of MD inputs is sufficient to elicit conditioned aversion in mice with chronic pain, but not in control mice, suggesting that the aversive signal transmitted by MD inputs to rACC is exacerbated in the setting of chronic pain. The enhancement of this aversive signal may be explained by differential recruitment of rACC output neurons in control vs. pain conditions. This possibility is examined in further detail in the next set of experiments.

Importantly, in a control experiment we confirmed that light delivery and virus injection alone into MD (driving expression of eYFP only) did not lead to conditioned preference or to aversion in nerve-injured animals (**Fig. 1–figure supplement D**). Thus, the behavioral effect we observe from activating MD inputs is not due to the effect of light.

Excitatory responses of layer V rACC pyramidal neurons evoked by MD inputs are significantly reduced in the setting of chronic neuropathic pain

We hypothesized that the ability of MD input activation to exacerbate pain-related aversion might reflect cellular and/or synaptic changes within the rACC. As we described in the Background section, Blom et al found that layer V rACC pyramidal neurons are hyperexcitable in a chronic nerve ligation model of neuropathic pain. Notably, the study did not distinguish between activity of the two distinct pyramidal cell populations that several reports have described within layer V of the rACC as well as neighboring subregions of the mPFC. One population projects to subcortical structures, including the MD (**Fig. 2A, B**).

These corticothalamic/subcortically-projecting (SC) cells exhibit a prominent hyperpolarization-activated cation current (I_h) that produces a prominent voltage sag and afterdepolarization in response to a hyperpolarizing current pulse (**Fig. 2C and D**). The second corticocallosal/intratelencephalically-projecting (IT) population projects to the contralateral cortex and has a much less pronounced sag/afterdepolarization (**Fig. 2**). Using these different physiological characteristics of IT and SC subtypes of pyramidal cells, we identified them in slices of the rACC and studied their intrinsic properties (**Fig. 3A, B).** As Figure 3C shows, we found that both populations of neurons recorded from slices of SNI and Taxol models of neuropathic pain were hyperexcitable compared to control, as indicated by a significant increase in the F/I slope. There was no change in the resting membrane potential or input resistance of either IT or SC neurons in the setting of pain, compared to control (**Fig. 3 – figure supplement A and B)**.

Next we asked whether MD inputs to the rACC, specifically to IT or SC neurons, are altered in the setting of chronic neuropathic pain. To evaluate this possibility, we again used AAV5-CaMKII-ChR2 virus to express ChR2-eYFP in projections from the MD to the rACC (**Fig. 4A, B**). Within the rACC, MD axons terminate in layer I and superficial layer V (**Fig. 4C**). To activate MD inputs we delivered trains of blue light flashes through a 40X objective while recording voltage clamp responses from IT or SC neurons (**Fig. 4D**). Traces of the population average of excitatory post-synaptic currents (EPSCs) evoked by 5Hz stimulation are shown in Figure 4E. To calculate the peak EPSC amplitudes, we averaged the

amplitude of the first EPSC in response to 5, 10, 20Hz trains of light. Surprisingly, we found that EPSCs elicited by MD input activation were dramatically reduced in both IT and SC neurons, in both SNI and Taxol animals compared to controls (**Fig. 4E and 4F**; $p < 0.05$ by unpaired t-test). In fact, despite the fact that both IT and SC neurons exhibit increases in intrinsic excitability, current clamp recordings showed that spiking in response to activation of MD inputs was, in fact, markedly reduced in both IT and SC cells from SNI animals compared to controls. In Taxol treated animals, we observed a similar reduction in IT cell spiking. In contrast, SC cells spiked at negligible levels in both Taxol and control mice (**Fig. 4–figure supplement**). Interestingly, the responses of IT cells to MD input activation were significantly greater than those of SC cells, in both animals with pain and controls ($p < 0.01$ by ANOVA using cell type, pain vs. control, and SNI vs. Taxol models as factors and including a possible interaction between cell type and condition). Thus MD inputs to rACC preferentially excite IT neurons, and are weakened in animals with pain (either SNI or Taxol) in both IT and SC neurons.

Chronic neuropathic pain shifts the E/I ratio elicited by MD input towards inhibition in SC neurons

We also recorded feedforward perisomatic inhibition evoked by MD inputs in IT and SC neurons, by recording in voltage clamp at +10mV while optogenetically activating MD inputs (**Fig. 5A**). Traces of the population average of feedforward inhibitory post-synaptic currents (IPSCs) evoked by 5Hz stimulation are shown in

Figure 5B. In contrast to the marked reduction we observed for excitatory responses to MD input activation in the setting of chronic pain, the amplitudes of feedforward IPSCs were not significantly different for SNI or Taxol compared to control animals (**Fig. 5C**). In particular, whereas reductions in EPSC amplitude were statistically significant for both SC and IT cells in both the SNI and Taxol models, none of these comparisons were statistically significant for feedforward IPSCs. For IPSCs in IT cells from SNI mice, the reduction in IPSC amplitude approached significance ($p = 0.052$, unpaired t-test), however these changes were not significant when we pooled together all of the IPSC measurements using an ANOVA with cell type, condition, and model as factors.

Given that we observed consistent reductions in EPSC amplitudes but more modest and inconsistent reductions in IPSCs, we explicitly tested whether the relative levels of excitation to inhibition (E:I ratio) elicited by MD-rACC inputs were altered in the setting of chronic pain. For each cell, we calculated E:I ratios based on the EPSCs and IPSCs measured during responses to MD input activation. Specifically, 1ms light pulses were determined to be the optimum pulse width to evoke EPSCs, while 5ms light pulses were determined to be optimum to evoke feedforward inhibition. We appreciate that a ratio of 1 does not imply perfectly balanced excitation and inhibition because EPSCs and IPSCs were recorded at different holding potentials and using slightly different conditions to optimize each set of measurements. Rather, by comparing E:I ratios, we could determine whether decreases in synaptic excitation are larger

than those in synaptic inhibition, when measured using a common set of cells from control and pain conditions. Indeed, we found that the E:I ratio for synaptic responses elicited by MD input activation was significantly smaller for animals with pain (i.e., shifted in the direction of inhibition) than for controls ($p < 0.05$ by ANOVA using pain vs. control, SNI vs. Taxol models, and cell type as factors) (**Fig. 5D**). This effect was present across both the SNI and Taxol models; however, the ANOVA did reveal a significant interaction between condition (pain vs. control) and cell type (IT vs. SC) ($p < 0.05$). Indeed, when examined separately, the E:I ratio was significantly reduced for SC but not IT neurons (**Fig. 5D**). This difference likely reflects the strong trend that we observed towards reduced IPSCs in IT neurons.

In order to further corroborate the MD-evoked inhibition of SC neurons in the setting of chronic pain, we performed an experiment in which we recorded spiking from an SC neuron in response to depolarizing current, with or without simultaneous optogenetic activation of MD inputs (**Fig. 6B**). We found that spiking of all control SC neurons was significantly increased by the addition of MD input activation (light ON) compared to current pulse alone (light OFF) ($p< 0.01$, ANOVA). In contrast, spiking of SC neurons from either SNI or Taxol animals was not significantly increased in light ON conditions. In fact, 4/6 SNI SC neurons actually showed reduced spiking when we activated MD inputs. In Taxol animals, some SC neurons showed a reduction in spiking, some did not change and some increased spiking in the light ON condition, compared to light OFF

(**Fig. 6A and C**). In summary, we consistently observed net excitation of SC neurons by MD input under control conditions, whereas in both pain models, MD inputs could actually reduce the firing of SC neurons. This adds further evidence that the net effect of MD input activation on SC neurons is shifted towards inhibition in the setting of chronic neuropathic pain.

Consistent with our finding that feedforward IPSCs were relatively intact in animals with pain, we also found that EPSCs recorded from fast-spiking interneurons did not differ between SNI and control animals (**Figure 7**). In other words, we found no evidence for disinhibition in the responses of layer V rACC pyramidal neurons to MD input in the setting of chronic pain.

DISCUSSION

Here we used optogenetics to activate a major source of input to the rACC – the mediodorsal thalamus (MD) – and uncovered surprising effects of this activation both *in vivo* and *in vitro*. We find that activation of MD inputs elicited conditioned place aversion in mice with chronic neuropathic pain, but not in control mice. To identify physiological changes within the rACC that may drive these behavioral effects, we recorded intrinsic properties of layer V rACC pyramidal neurons as well as their responses to optogenetic activation of MD inputs to the rACC. We focused on two subtypes of layer V rACC pyramidal projection neurons: intratelencephically-projecting (IT) and subcortically-projecting (SC). Both IT and SC neurons are hyperexcitable in the setting of chronic neuropathic pain; in spite of this, their excitatory responses evoked by MD inputs decreases significantly in both SNI and Taxol models of chronic neuropathic pain. Additionally, we found that MD inputs preferentially recruit IT neurons and that the E/I balance of SC neurons responses to MD inputs shifts towards inhibition in the setting of chronic neuropathic pain. In the next chapters, we will explore the significance of these results and test models in which pain-related aversion is differentially regulated by the activity of subsets of layer V rACC pyramidal neurons.

The contribution of MD thalamus to chronic pain

The MD, like the rACC, is heavily connected with limbic regions, including the prefrontal and insular cortex, striatum and the amygdala (Krettek and Price, 1977; Ray and Price, 1992; Velayos et al., 1985). As a result, the MD is

positioned to integrate and influence nociceptive, cognitive, and affective processing streams. It is, therefore, not surprising that the MD has been implicated in a number of cognitive processes, including fear conditioning (Li et al., 2004; Lee et al., 2011), instrumental learning (Floresco et al., 1999; Ostlund and Balleine, 2008; Mitchell and Chakraborthy, 2013), and discriminative avoidance learning (Smith et al., 2002; Gabriel et al., 1989). The MD is also a critical structure linked to many neurological disorders including schizophrenia, major depressive disorder, Parkinson's and Alzheimer's disease (Mitchell and Chakraborthy, 2013).

Many previous studies of the contribution of MD to pain processing examined the effect of MD lesions or pharmacological blockade of MD function. Such studies, however, are limited, as the approaches do not allow for the study of MD connections to other brain regions. In fact, lesion studies of the MD and pain-related aversion have been largely inconclusive, due to differences in the size and location of the lesions, as well as the variable atrophy of surrounding target structures. The latter is a serious concern because of the complexity and proximity of medial thalamic nuclei (Wilson et al., 2008; Mitchell and Chakraborthy, 2013). For example, in CPA paradigms, excitotoxic lesions of medial thalamic nuclei did not alter conditioned avoidance in an animal model of visceral pain (Wang et al., 2007), but in another paradigm, did block inhibitory avoidance learning to a footshock (Li et al., 2004; Chai et al., 2010). Electrophysiological studies of the connections MD, including those with the

rACC have faced similar limitations in specificity. Although brain sections that preserve the thalamocingulate pathway can be prepared, electrical stimulation still activates fibers of passage and axon collaterals, which confounds the specificity of responses evoked in the rACC (Lee et al., 2007). Optogenetic techniques have made it possible to overcome some of these limitations, enabling a closer and more specific delineation of MD-rACC interactions. Our study provides new information concerning the ability of MD inputs to influence representations of pain-related aversion in the rACC.

MD inputs and layer V rACC SC neurons form an inhibitory circuit involved in pain-related aversion

Feedforward inhibition mediated by cortical inhibitory interneurons (Agmon and Connors, 1991; Swadlow, 2002; Swadlow, 2003) is a key feature of thalamocortical interactions is and is thought to control the temporal precision of cortical responses to sensory stimuli. MD inputs directly innervate inhibitory interneurons in the rACC, particularly those that express parvalbumin (PV) (Delevich et al., 2015). Silencing local PV interneurons in the rACC increases the duration, but not amplitude, of MD-evoked EPSCs in pyramidal neurons of the rACC, which suggests that PV-mediated feedforward inhibition in rACC controls temporal integration (Delevich et al., 2015). In our studies, we recorded strong feedforward inhibition of SC neurons by MD inputs in the setting of chronic pain. Conceivably this inhibition affects the temporal integration of responses of SC neurons evoked by other inputs, effectively serving as a gate that regulates

integrated input to the rACC. In fact, there is anatomical evidence that MD inputs to the rACC are positioned to execute this type of gating. Specifically, MD afferents terminate in layer V and superficially in layer 1. As PV-positive inhibitory interneurons reside in layer V of the rACC (Kalus and Senitz, 1996), the MD inputs can recruit feedforward inhibition at the level of the soma of layer V pyramidal rACC neurons. Moreover, ultrastructural studies have demonstrated that MD inputs form synapses with spines along the length of the apical dendrite of layer V rACC pyramidal neurons (Kuroda et al., 1998; Berendse and Groenewegen, 1991). As these apical dendrites and their branches project into superficial layers, they are well-positioned to sample diverse inputs, for example, from the basolateral amygdala (BLA) or ventral hippocampus (Hoover and Vertes, 2007). Indeed, it has been shown that MD input activation exerts powerful inhibitory gating of hippocampal-evoked firing of layer V pyramidal neurons in neighboring regions of the mPFC (Floresco and Grace, 2003).

There is also anatomical and electrophysiological evidence for a recurrent excitatory loop between the MD and rACC. For example, ultrastructural studies demonstrate that thalamocortical axon terminals from MD make direct excitatory synapses with dendrites of rACC pyramidal neurons that project back to medial thalamic nuclei, including the MD (Kuroda et al., 1993a). Electrophysiological studies demonstrated that high frequency electrical stimulation of the MD can evoke longer latency spikes in the rACC and neighboring regions of the mPFC, likely by antidromic activation of glutamatergic axon collaterals of output

neurons that project to the MD (Pirot et al., 1994). We suggest that inhibition of SC output function, which we measured by a reduction of spiking in the setting of chronic pain, affects activity within this loop. For example, assuming that the loop supports neural synchrony between the MD and rACC, then a reduction in corticothalamic tone could disrupt the transmission of relevant affective and nociceptive information. Indeed, several studies demonstrated that task performance depends on thalamocortical synchrony. For example, chemogenetic inhibition of MD disrupted MD-mPFC synchrony and impaired performance on mPFC-dependent working memory task (Parnadeau et al., 2013).

In the next chapter, we will examine the effects of activating another set of inputs to the rACC, in order to test if there is input-specific modulation of rACC activity and subsequent representations of pain-related aversion.

METHODS

All experiments were conducted in accordance with procedures established by the Administrative Panels on Laboratory Animal Care at the University of California, San Francisco.

Animals. All experiments were performed with male C57BL/6J mice (Simonsen Laboratory). Animals were group-housed under standard conditions with *ad libitum* access to food and water. For all electrophysiology experiments all animals were housed under a standard 12 hr light/dark cycle. For behavioral experiments conducted in red light, animals were housed under a reverse 12 hr light/dark cycle for 3 days prior to and for the duration of the experiment. Sample sizes were chosen based on effect sizes from previous experiments.

Slice preparation. We cut 250 μm coronal slices from 8 to 10-week-old male C57BL6 mice (Gee et al., 2012). Slices were cut in a chilled slicing solution in which Na+ was replaced by sucrose, then incubated in warmed artificial cerebrospinal fluid (ACSF) at 30°C–31°C for ~1 hr before being used for recordings. ACSF contained 126 mM NaCl, 26 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl, and 10 mM glucose. We secured the slice by placing a harp along the midline between the two hemispheres.

Intracellular recording. We obtained somatic whole-cell patch recordings from visually identified pyramidal cells in layer V of rostral anterior cingulate cortex using differential contrast video microscopy on an upright microscope (BX51WI; Olympus). Recordings were made using a Multiclamp 700A (Molecular

Devices). Except when otherwise noted, patch electrodes (tip resistance = 2–6 MOhms) were filled with the following (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 EGTA, 2 MgCl, 2 MgATP, and 0.3 NaGTP (pH adjusted to 7.3 with KOH). All recordings were at 32.5±1°C. Series resistance was usually 10–20 MΩ, and experiments were discontinued above 30 MΩ.

Voltage clamp recordings: To record EPSCs, we held cells at -70 mV and recorded responses to MD inputs evoked by 1.0 ms light pulses. 1.0 ms was chosen as the optimum pulse width, as we observed that excitation evoked by MD inputs was so strong that sometimes unclamped spikes were elicited even in voltage clamp. To record feedforward inhibition, we held the same cells at +10 mV and recorded responses to 5.0 ms light pulses. In contrast to excitation, 1.0 ms light pulses were not strong enough to evoke responses and thus 5.0 ms light pulses were determined to be optimum to evoke feedforward inhibition.

Current clamp recordings: To record the effect of MD input activation on spiking, we recorded neurons in current clamp mode with the same intracellular solution. In a separate experiment to identify MD-evoked inhibition of SC neurons, we again recorded in current clamp mode, whereby we delivered a 1.0 sec current pulse (starting at 50 pA and increasing in 50 pA steps), a 1.0 sec gap, and then a second current pulse. 20 Hz, 1.0 ms blue light flashes came on 100 ms before the second current pulse and turned off at the end of the current pulse. There was a 10 sec delay before the next sweep.

Expression of optogenetic proteins: We expressed channelrhodopsin (ChR2) in terminals from MD using an AAV5 vector that contains a gene

encoding ChR2-eYFP under control of the CaMKIIα promoter. We expressed archaerhodopsin from MD terminals using an AAV5 vector that contains a gene encoding eArch3.0-eYFP under the control of the Synapsin promoter. We injected a control virus containing eYFP alone under control of the CaMKII promoter for stimulation experiments. All adeno-associated viruses (AAV) were designed in the Deisseroth Lab and purchased from UNC Vector Core. For injections into MD thalamus, coordinates were (in millimeters relative to bregma): -1.7 A/P, +0.3 M/L, and -3.43 D/V. All injections were made in 3-4 week old male C57BL6 animals, and we waited 4-6 weeks after virus injection before preparing brain slices.

***In vitro* ChR2 stimulation.** We stimulated ChR2 in MD terminals using flashes of light generated by a Lambda DG-4 high-speed optical switch with a 300W Xenon lamp, and an excitation filter set centered around 470 nm, delivered to the slice through a 40X objective (Olympus). Illumination was delivered across a full high-power (40X) field.

***In vivo* optogenetic stimulation.** For all behavioral experiments, we injected mice with a ChR2-eYFP or eYFP virus and implanted them with a chronic unilateral custom-made optical fiber targeted to the rACC (+1.51mm A/P, +0.25mm M/L, -1.13mm D/V relative to Bregma). We connected mice to a 'dummy' optical patch cable 2 days before the experiment each day for 30–60 min to habituate them to the tethering procedure. Following the tethering procedure, we ran mice in the behavioral procedure (see below). For channelrhodopsin stimulation experiments, we used a 10 mW laser with a

stimulation frequency of 20 Hz and a 5.0 ms light pulse duration for all behavioral experiments. Total light power for all experiments was 5-10 mW. For archaerhodopsin experiments, we delivered continuous stimulation at a power of 2-2.5 mW.

Retrograde labeling: For retrograde labeling of IT and SC cells, we injected Alexa-Fluor conjugated cholera toxin beta-subunit (AF-CTB) 488 nm and 594 nm (Invitrogen) into the MD thalamus and contralateral cortex (+1.50 A/P, +0.3 M/L, -1.60 D/V measured in millimeters, relative to Bregma).

Neuropathic pain models: Chemotherapy (Taxol)-induced neuropathy and the spared nerve injury (SNI) model were used. Four intraperitoneal injections (i.p) of Taxol (diluted in 40% DMSO) at a dose of 1mg/kg were made every other day. Control animals received saline injections. In the SNI model, the tibial branch of the sciatic nerve was isolated, and then 8-0 silk suture was used to ligate the sural and common peroneal branches of the sciatic. Next the ligated branches were transected distal to the ligature and 2-4 mm of each distal nerve stump removed. Control animals had no surgery. For all physiology experiments, the experimenter had a colleague kill the animal so that experimenter was blind to the eartag and genotype (pain or no pain) before slicing. For all behavior experiments, a colleague did all of the Taxol and SNI surgeries respectively and the code was broken at the time of data analysis. Each cage of mice included both experimental and control mice.

Conditioned Place Preference. The conditioned place preference (CPP) apparatus consists of a three-chambered box, a left chamber with vertical black

and white stripes on the wall and a punched metal floor, a middle neutral chamber with grey walls and a smooth floor, and a right chamber with black and white spots on the wall and a mesh floor. The CPP test consists of 4 days. Day 1 is the Pre-Test Day in which mice have access to all three chambers, and we record the time spent in each chamber. On days 2 and 3, animals are restricted to either the left or right chamber (counterbalanced across all mice) and receive either 5.0 ms flashes of blue light at 20 Hz (stimulation) for 20 min, or no stimulation for 20 min. Approximately 4 hours later, the mice are restricted to the opposite chamber and receive the other treatment (stimulation or no stimulation). 24h after the last conditioning session on Day 4 – Test Day – the mice again have access to all three chambers, and time spent in each chamber is recorded. We calculated a Preference Score for the stimulated chamber (Preference for Stim Side - the ratio of the time spent in the stimulated side on Test day to Pre-Test day. We also calculated a Difference Score (The time spent in the stimulated chamber on Pre-Test day subtracted from the time spent in that chamber on Test day). Animals were not used if they spent more than 75% of total time spent in one chamber on Pre-Test day.

Statistical analysis. We used Student's t-tests (two-tailed, unpaired) to compare pairs of groups, unless there were repeated measurements or unpaired observations, in which case we used ANOVA. Automatic tests in Prism were used to confirm normality and suitability for t-tests. All of the data are represented as means with standard error of the mean. Outliers were defined as three standard deviations away from the mean.

Figure 1: Optogenetic activation of MD inputs to rACC exacerbates pain aversion in SNI and Taxol animals, but has no effect on control animals.
A. Conditioned Place Preference (CPP) Paradigm. **B.** (Left) Timeline of injection, implantation and behavior. (Right) AAV5-CaMKII ChR2-eYFP injection in MD thalamus and light fiber in rACC. **C.** Preference for Stim Side quantifies the ratio of the time spent in the stimulated chamber on Test day to Pre-test day. A score of 1 means there was no effect of conditioning. A score > 1 indicates that the stimulation was less aversive (< aversive), while a score < 1 indicates that the stimulation was more aversive (> aversive). SNI (n=13) and Taxol (n=10) animals have preference scores significantly < 1 (*, $p < 0.05$ by unpaired t-test.), i.e., they avoid the side paired with optogenetic activation of MD inputs. Preference scores of control (n=11) and saline (n=9) animals are not significantly different from 1. Error bars represent S.E.M. **D.** AAV5-Synapsin-Arch-eYFP injection in MD and optical inhibition of MD terminals in rACC. **E.** Quantification of Preference for Stim Side of control (n=6) and SNI (n=5) animals. Both groups have a score > 1 (*, $p \leq 0.05$ by unpaired t-test.), indicating that both groups prefer the side paired with optogenetic inhibition of MD inputs. The difference in the preference score between control and SNI animals is not statistically significant. Error bars represent S.E.M.

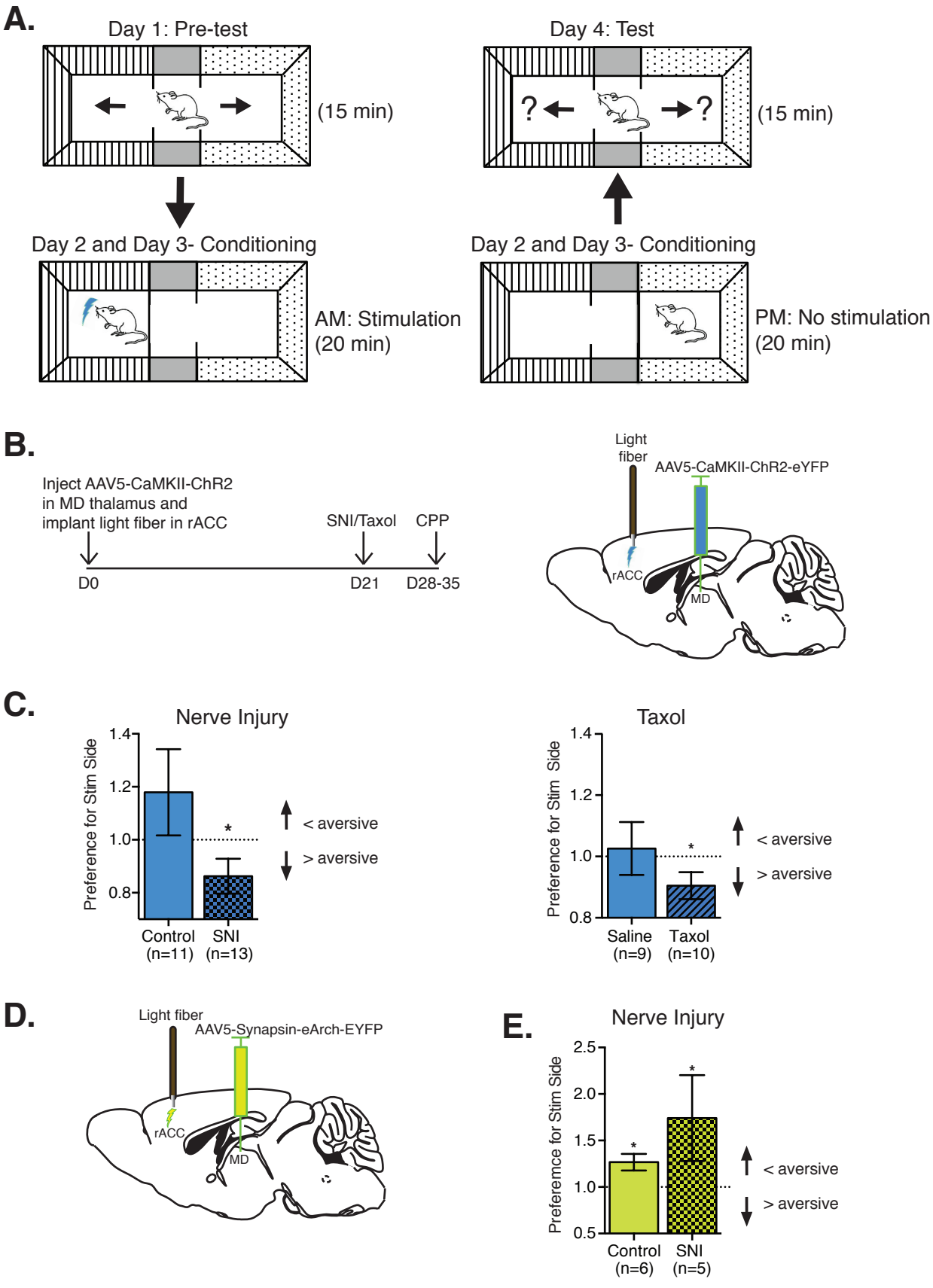
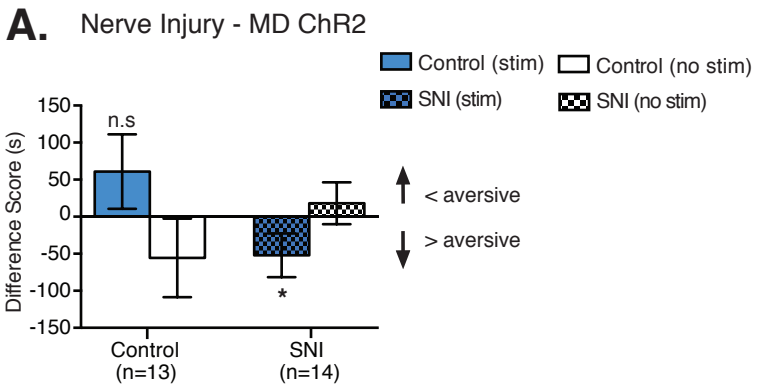
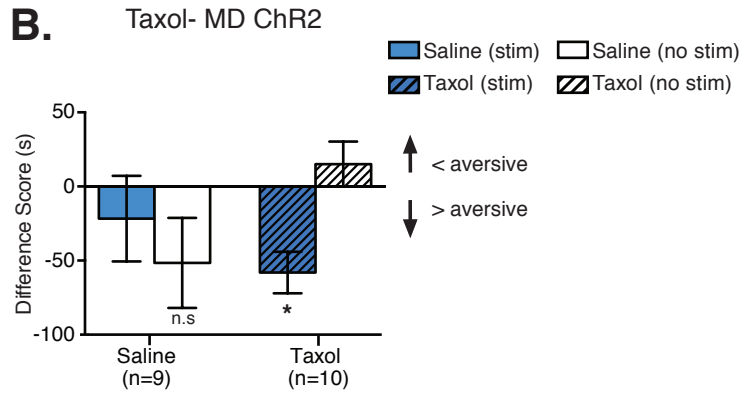
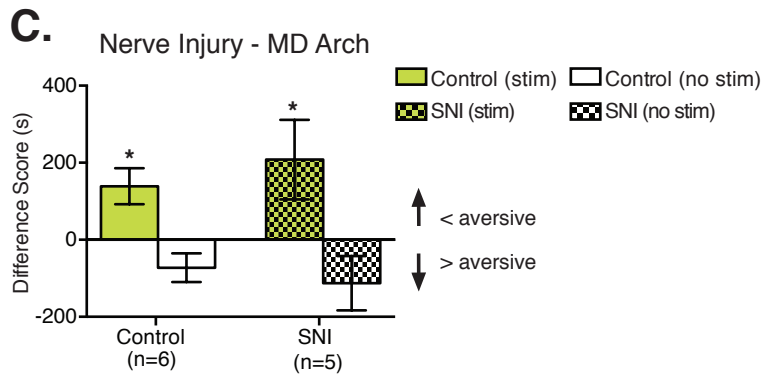

A.
Day 1: Pre-test
(15 min)
Day 4: Test
(15 min)
Day 2 and Day 3- Conditioning
AM: Stimulation
(20 min)
Day 2 and Day 3- Conditioning
PM: No stimulation
(20 min)
B.
Inject AAV5-CaMKII-ChR2
in MD thalamus and
implant light fiber in rACC
SNI/Taxol
CPP
D0
D21
D28-35
Light
fiber
AAV5-CaMKII-ChR2-eYFP
rACC
MD
C.
Nerve Injury
Preference for Stim Side
1.4
1.2
1.0
0.8
< aversive
> aversive
Control
(n=11)
SNI
(n=13)
Taxol
Preference for Stim Side
1.4
1.2
1.0
0.8
< aversive
> aversive
Saline
(n=9)
Taxol
(n=10)
D.
Light fiber
AAV5-Synapsin-eArch-EYFP
rACC
MD
E.
Nerve Injury
Preferemce for Stim Side
2.5
2.0
1.5
1.0
0.5
< aversive
> aversive
Control
(n=6)
SNI
(n=5)

Figure 1–figure supplement: (A-C) CPP data presented as Difference Scores. (D) There is no effect of 473nm light on injured animals injected with control AAV5-CaMKII-eYFP virus in MD.

A and B. For animals receiving optogenetic activation of MD inputs to the rACC, the Difference Score quantifies the difference in time (Test - Pre-test) spent in the stimulated or unstimulated chambers. A difference score of 0 means there was no effect of conditioning. A difference score < 0 means conditioning was more aversive, while a difference score > 0 means conditioning was less aversive/induces preference. SNI and Taxol animals have Difference Scores significantly < 0 for the stimulated side, meaning they spend less time on the side paired with optogenetic activation of MD inputs. No such effect occurred in control animals. *, $p<0.05$ by t-test. Error bars represent S.E.M. **C.** Control (pain free) and SNI animals both have Difference Score to the stimulated side > 0, meaning both groups preferred the chamber paired with optogenetic inhibition of MD inputs. There is no difference in the Difference Score for the stimulated side between control and SNI animals. Error bars represent S.E.M. **D.** SNI animals injected with a control AAV5-CaMKII-eYFP virus in the MD did not develop preference or aversion to the stimulated side, showing there is no effect of blue light alone. Error bars represent S.E.M.


A.
Nerve Injury - MD ChR2
Control (stim)
Control (no stim)
SNI (stim)
SNI (no stim)
Difference Score (s)
150
100
50
0
-50
-100
-150
n.s
*
< aversive
> aversive
Control
(n=13)
SNI
(n=14)


B.
Taxol- MD ChR2
Saline (stim)
Saline (no stim)
Taxol (stim)
Taxol (no stim)
Difference Score (s)
50
0
-50
-100
n.s
*
< aversive
> aversive
Saline
(n=9)
Taxol
(n=10)


C.
Nerve Injury - MD Arch
Control (stim)
Control (no stim)
SNI (stim)
SNI (no stim)
Difference Score (s)
400
200
0
-200
*
*
< aversive
> aversive
Control
(n=6)
SNI
(n=5)

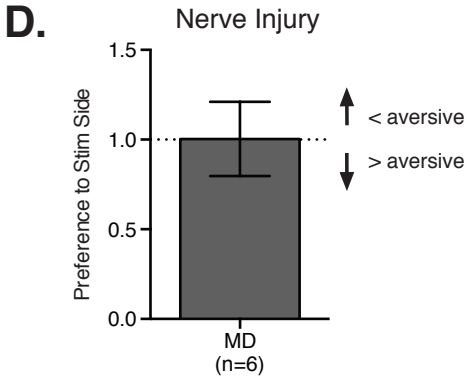

D.
Nerve Injury
Preference to Stim Side
1.5
1.0
0.5
0.0
< aversive
> aversive
MD
(n=6)

Figure 2: Subtypes of layer V rACC pyramidal neurons differ in their projection targets and in the prominence of hyperpolarization activated h-current (I_h)

A. Schematic of AF-CTB injections in the contralateral cortex or mediodorsal thalamus to label corticocallosal/intratelencephalic (IT) and corticothalamic/ subcortical (SC) projecting cells respectively. **B.** (Left) Low power image of retrogradely labeled IT cells (red) and SC cells (green) in Cg1/rACC. Midline and the layers are demarcated. Scale bar = 50 μm. (Right) High power image of inset shows overlap of IT and SC cells in Cg1/rACC. Scale bar = 10 μm. **C.** Sample recordings from labeled IT and SC neurons in response to hyperpolarizing or depolarizing current injection. The voltage sag and rebound after-depolarization (ADP) in response to hyperpolarizing current injection is evident in the SC neuron, but not the IT neuron (arrows). **D.** The magnitude of h-current, measured as the sum of the voltage sag and rebound afterdepolarization, in response to hyperpolarizing current pulses in IT and SC neurons. Numbers of cells are indicated in parentheses. Error bars represent S.E.M.

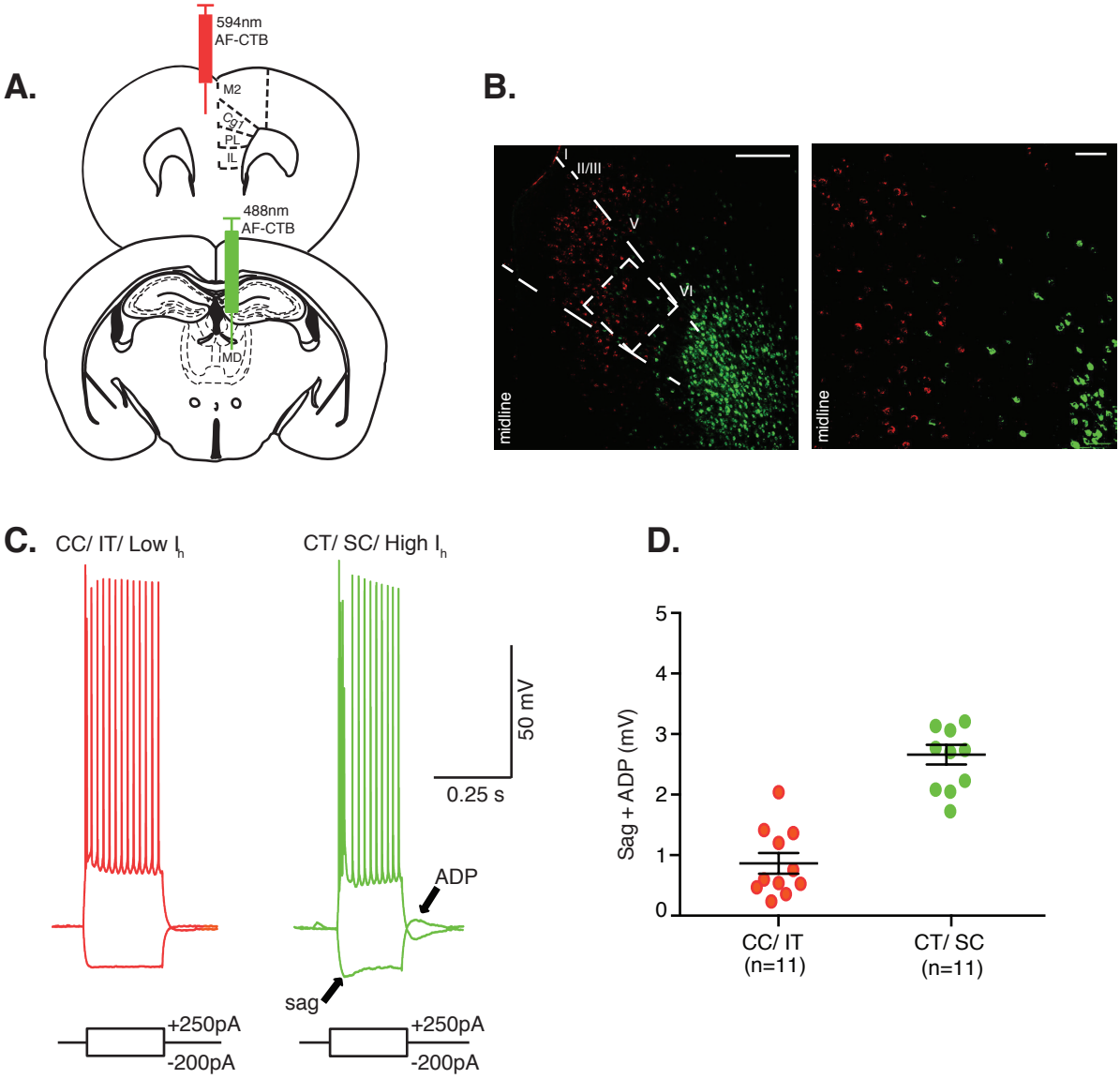

A.
594nm
AF-CTB
M2
Cg1
PL
IL
488nm
AF-CTB
MD
B.
I
II/III
V
VI
midline
midline
C.
CC/ IT/ Low I h
CT/ SC/ High I h
50 mV
0.25 s
ADP
sag
+250pA
-200pA
+250pA
-200pA
D.
Sag + ADP (mV)
5
4
3
2
1
0
CC/ IT
(n=11)
CT/ SC
(n=11)

Figure 3: IT and SC subtypes of layer V rACC pyramidal cells are hyperexcitable in SNI and Taxol animals compared to controls animals.
A. Top: Representative coronal sections from Bregma 1.48 mm to Bregma 1.18 mm including rACC (Cg1 and Cg2). Bottom left: DIC image of patch pipette in layer V of rACC. Scale bar = 50 μm. **B.** Bottom right: 40X image of whole-cell patch clamp recording. **C.** The F/I slope (Hz/pA) was significantly increased in both cell types from SNI (n=3) and Taxol (Tax) animals (n=3) compared to controls (n=3). The number of cells for each experiment is indicated in parentheses. *, $p < 0.05$; **, $p < 0.01$ by unpaired t-test. Error bars represent S.E.M.

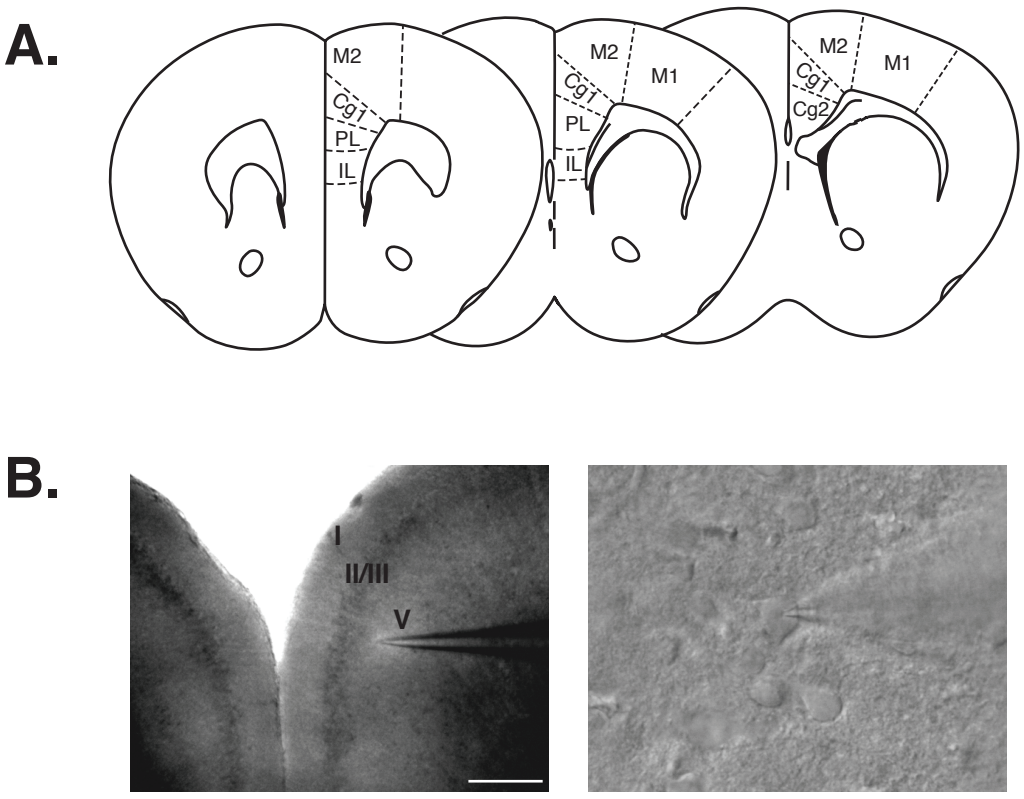

A.
M2
Cg1
PL
IL
M1
Cg2
B.
I
II/III
V
C.
Nerve Injury
Taxol
F/I slope (Hz / pA)
F/I slope (Hz / sec)
Con
SNI
Sal
Tax
(21)
(27)
(11)
(20)
(22)
(12)
IT
SC

Figure 3 – figure supplement: Resting membrane potential and input resistance of layer V rACC pyramidal neurons are not changed in SNI or Taxol animals compared to control.

A. V_{rest} (resting membrane potential) in IT and SC cells (number of cells indicated in parentheses). There is no difference between SNI (n = 3 mice) or Taxol conditions (n = 3) and controls (n = 3). **B.** R_{input} (input resistance) in IT and SC cells (number of cells indicated in parentheses); does not differ between SNI (n = 3 mice) or Taxol mice (n=3) compared to controls (n = 3).

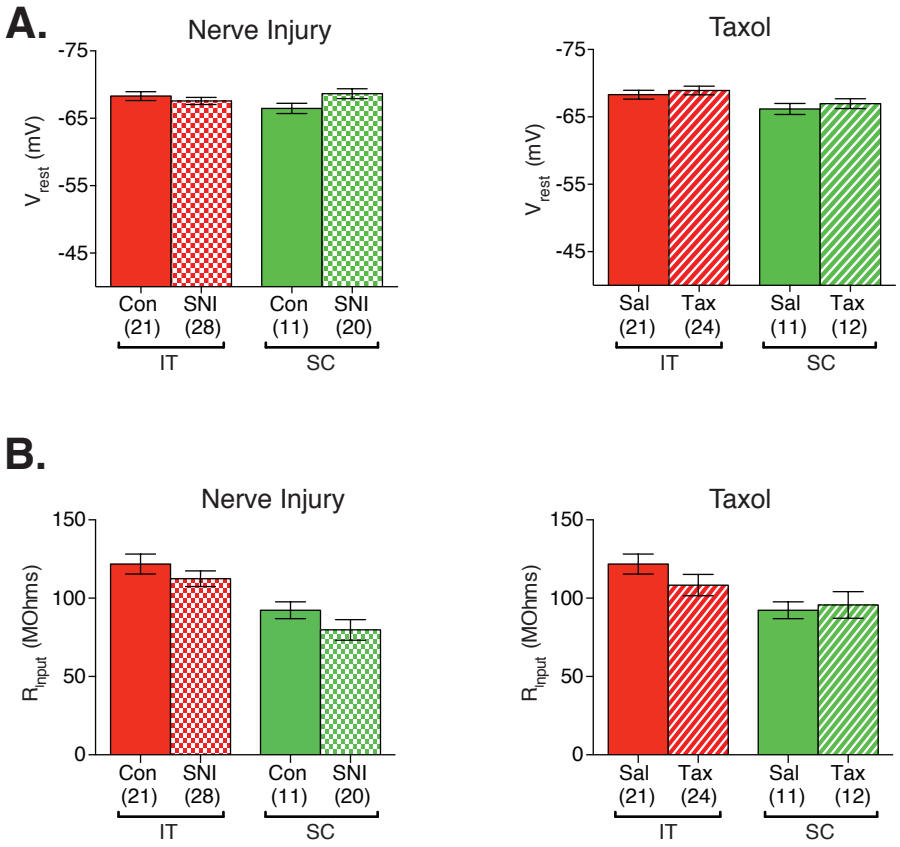

A.
Nerve Injury
Taxol
-75
-65
-55
-45
V rest (mV)
Con (21)
SNI (28)
Con (11)
SNI (20)
IT
SC
Sal (21)
Tax (24)
Sal (11)
Tax (12)
B.
150
100
50
0
R Input (MOhms)

Figure 4: Excitatory responses of layer V rACC pyramidal neurons to optogenetic activation of MD inputs are reduced in both SNI and Taxol animals compared to controls.

A. Coronal section of a mouse brain at Bregma -1.70 mm shows the site of AAV5-CaMKII-Chr2-eYFP injection in the MD thalamus. **B.** Image of fluorescent labeling of the targeted MD. “3v” indicates the location of the 3rd ventricle. Scale bar = 50 µm. **C.** ChR2-eYFP expression in layer I and layer V of the rACC.

D. Schematic illustrating whole-cell patch clamp recordings of responses of IT and SC cells to activation of ChR2-eYFP expressing inputs (yellow) from the MD thalamus. **E.** Population average of excitatory post-synaptic currents (EPSCs) (voltage clamp responses at -70 mV) to 5.0 Hz trains of 1.0 ms light pulses recorded from IT and SC cells of SNI (n = 4 mice) vs. control mice (n = 4) and Taxol (n = 3) vs. saline-treated mice (n = 3). A zoom-in of the first light-evoked response is shown in the insets. **F.** The amplitude of the first EPSC during 5, 10, or 20 Hz trains is averaged to calculate the peak EPSC amplitude for each cell. Quantification of the peak EPSC amplitude in IT and SC cells (number of cells in each experiment indicated in parentheses) from SNI or Taxol animals compared to controls. *, $p < 0.05$ by unpaired t-test. Error bars represent S.E.M.

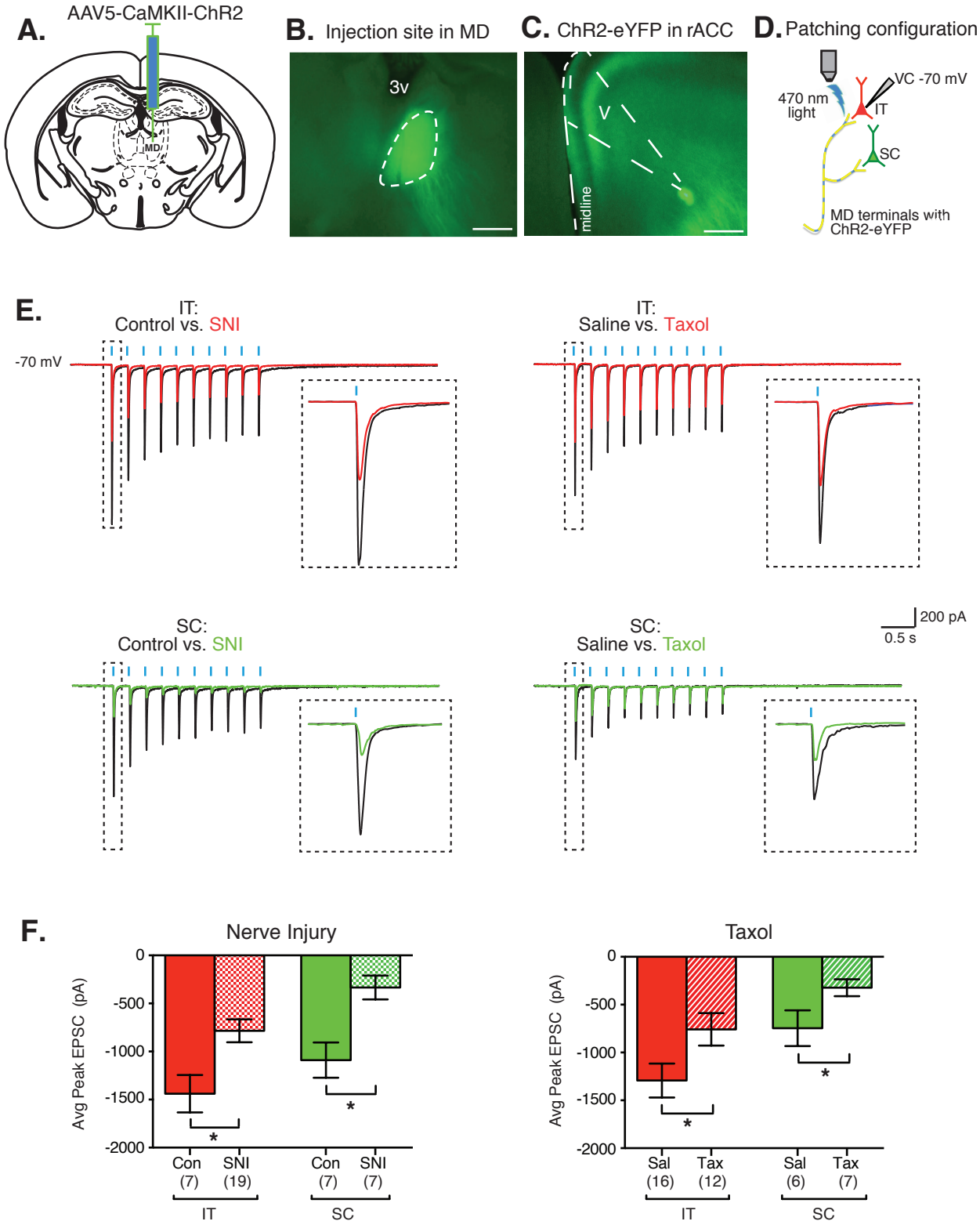

A.
AAV5-CaMKII-ChR2
MD
B. Injection site in MD
3v
C. ChR2-eYFP in rACC
V
midline
D. Patching configuration
470 nm light
VC -70 mV
IT
SC
MD terminals with ChR2-eYFP
E.
IT: Control vs. SNI
-70 mV
IT: Saline vs. Taxol
SC: Control vs. SNI
SC: Saline vs. Taxol
200 pA
0.5 s
F.
Nerve Injury
Avg Peak EPSC (pA)
0
-500
-1000
-1500
-2000
Con (7)
SNI (19)
Con (7)
SNI (7)
IT
SC
Taxol
Sal (16)
Tax (12)
Sal (6)
Tax (7)

Figure 4–figure supplement: Spiking of layer V rACC pyramidal cells evoked by optogenetic activation of MD inputs is significantly reduced in the setting of persistent neuropathic pain.
A. Recording configuration. **B.** Example of current clamp responses. The spike probability is the number of light pulses that evoked spikes divided by the total number of light pulses. **C.** Population average of current clamp responses of IT or SC cells (number of cells in parentheses) to optogenetic stimulation of MD inputs in SNI (n = 4) or Taxol animals (n = 3) compared to controls animals (n = 4 in each case). *, $p < 0.05$, ** , $p < 0.01$ by unpaired t-test. Error bars represent S.E.M.

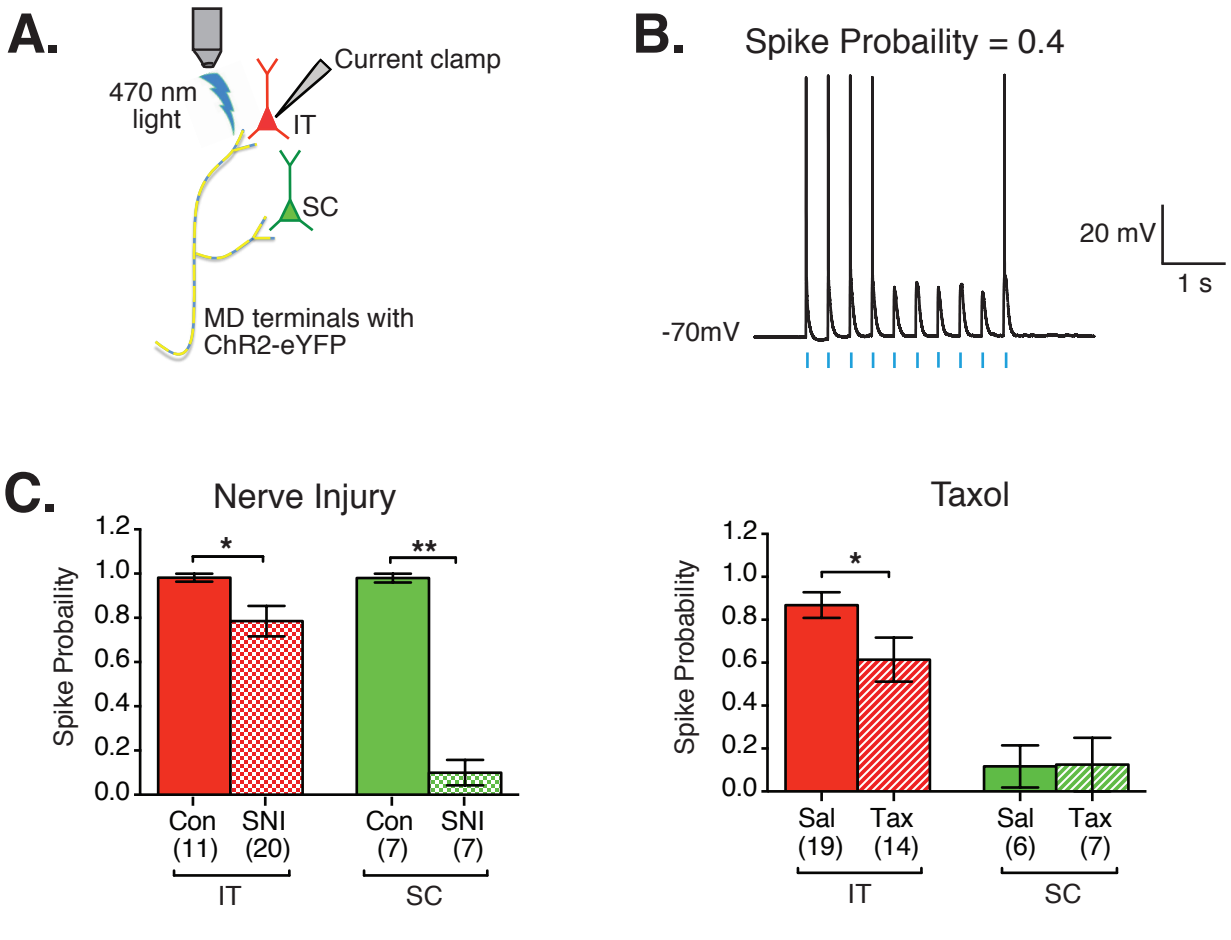

A.
470 nm
light
Current clamp
IT
SC
MD terminals with
ChR2-eYFP
B.
Spike Probaility = 0.4
20 mV
1 s
-70mV
C.
Nerve Injury
Spike Probability
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Con
(11)
SNI
(20)
Con
(7)
SNI
(7)
IT
SC
Taxol
Spike Probability
*
Sal
(19)
Tax
(14)
Sal
(6)
Tax
(7)
IT
SC

Figure 5: Feedforward inhibition evoked by MD inputs is maintained in the setting of chronic pain, resulting in a shift of the E/I ratio towards inhibition for SC neurons within the rACC.
A. Schematic of patching configuration to record feedforward inhibition from IT or SC neurons. **B.** Population average of inhibitory postsynaptic currents (IPSCs) to 5.0 Hz trains of 5.0 ms light pulses recorded in voltage clamp at +10 mV from IT and SC cells. A zoom in of the first light-evoked response is shown in the insets. **C.** Quantification of peak IPSC amplitude recorded in voltage clamp at +10mV from IT and SC cells (number of cells indicated in parentheses) in SNI animals (n = 4 mice) or Taxol animals (n = 3) compared to controls (n = 3 in each case). Error bars represent S.E.M. **D.** The area under the curve (AUC) for EPSCs and IPSCs (recorded in voltage clamp at -70 or +10 mV, respectively) was calculated for responses to 10 stimuli delivered at 5.0 Hz. In each case, we plotted the ratio of the excitatory to inhibitory AUC for all IT and SC neurons in SNI or Taxol vs. control. *, $p<0.05$ using two-way ANOVA with a main effects of SNI vs. Taxol and pain vs. control, but no interaction. Error bars represent S.E.M.

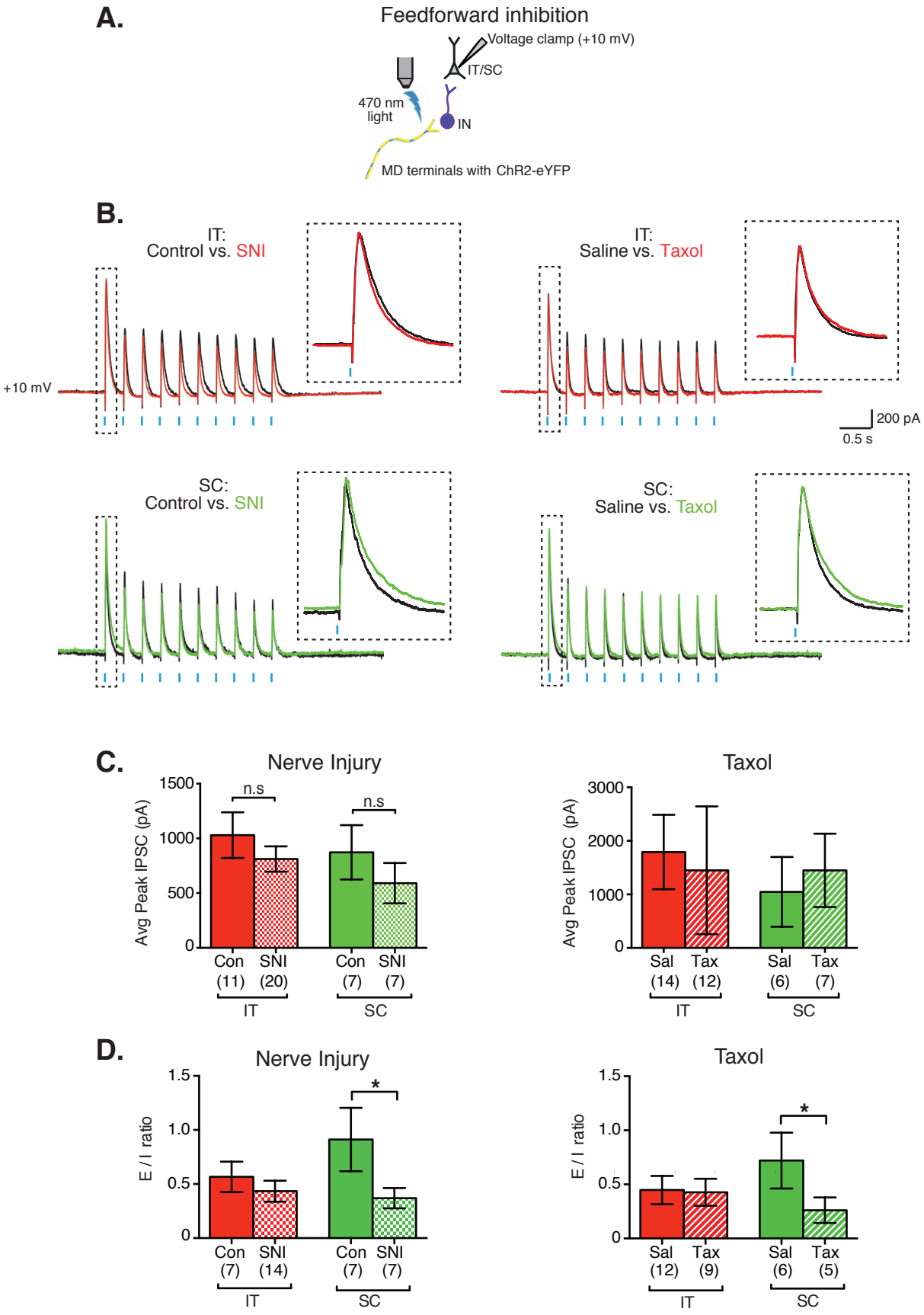

A.
Feedforward inhibition
Voltage clamp (+10 mV)
IT/SC
470 nm light
IN
MD terminals with ChR2-eYFP
B.
IT: Control vs. SNI
IT: Saline vs. Taxol
+10 mV
200 pA
0.5 s
SC: Control vs. SNI
SC: Saline vs. Taxol
C.
Nerve Injury
Taxol
Avg Peak IPSC (pA)
n.s
n.s
Con (11) SNI (20) Con (7) SNI (7)
IT
SC
Sal (14) Tax (12) Sal (6) Tax (7)
D.
Nerve Injury
Taxol
E / I ratio
*
Con (7) SNI (14) Con (7) SNI (7)
Sal (12) Tax (9) Sal (6) Tax (5)

Figure 6: Spiking of layer V SC neurons in response to a depolarizing current pulse combined with optogenetic activation of MD input is significantly increased only in control animals.
A. Example traces of spiking of Control (top), Taxol (middle), and SNI (bottom) in response to the depolarizing current pulse with and without optogenetic stimulation. **B.** Traces of current pulse protocol and optogenetic stimulation protocol. **C.** Number of spikes of cells from Control (black circles), SNI (red triangles), and Taxol (blue inverted triangles) plotted as a function of depolarizing current pulse with optogenetic activation of MD inputs (light ON, y-axis) and without optogenetic activation (light OFF, x-axis). The unity line indicates that there's no change in spiking between light ON and light OFF conditions. All of the Control cells (black circles) lie above the unity line, indicating that spiking significantly increases in the light ON conditions. The majority of SNI and Taxol cells lie below the unity line, indicating that optogenetic activation of MD inputs combined with depolarizing current pulse does not significantly increase spiking.

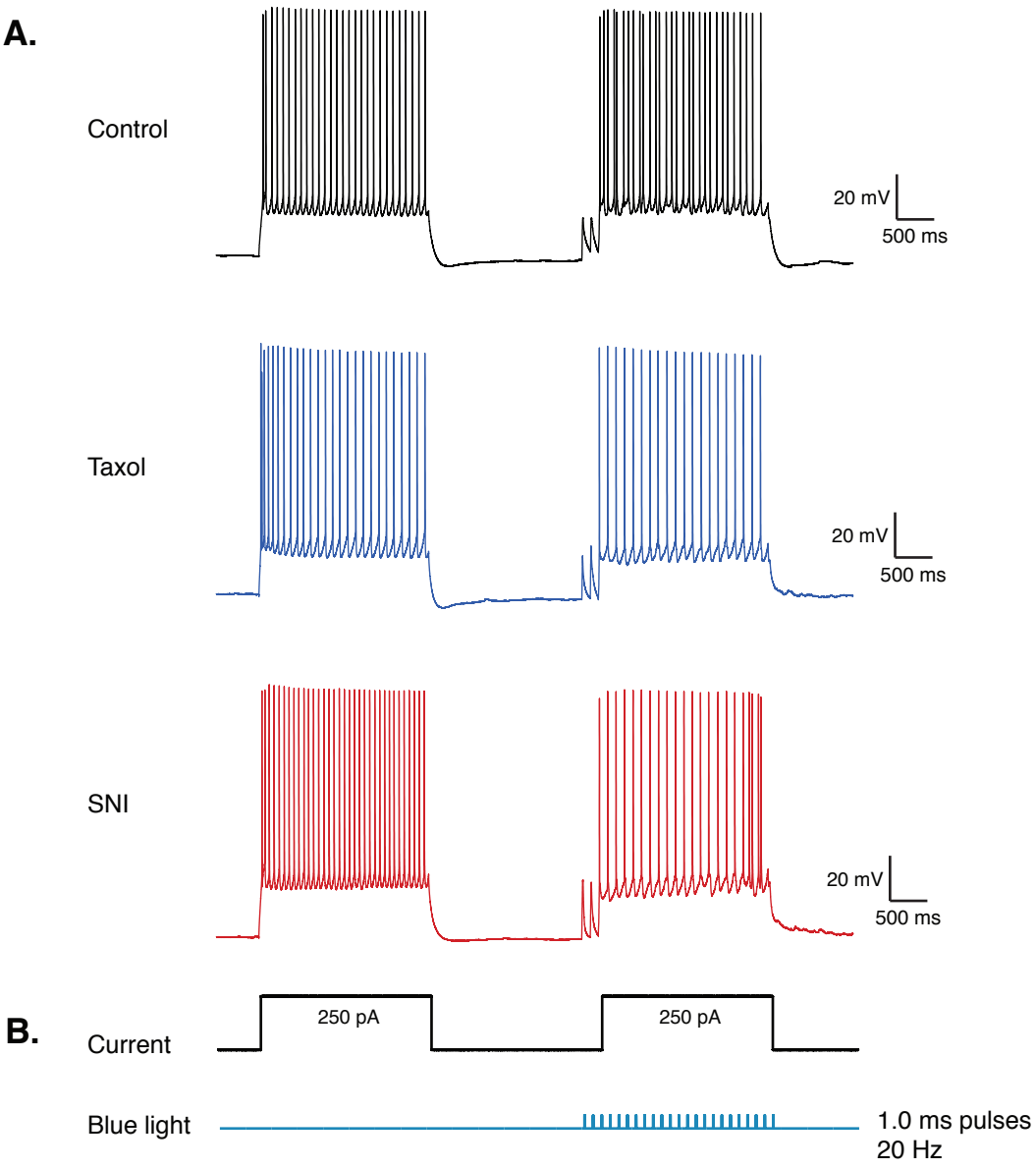
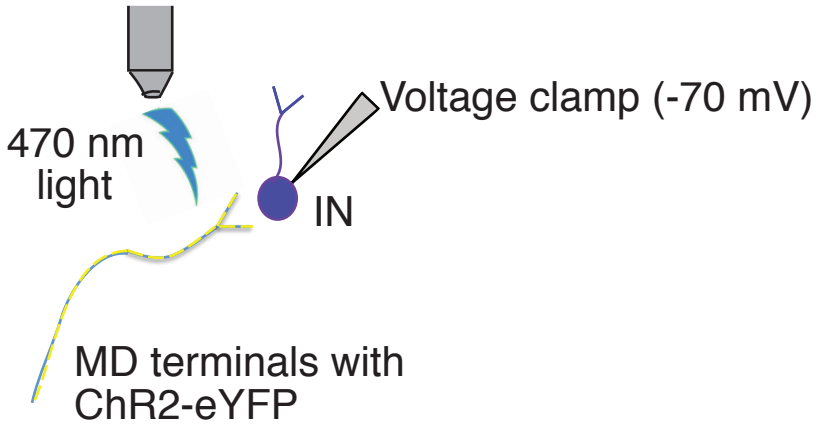
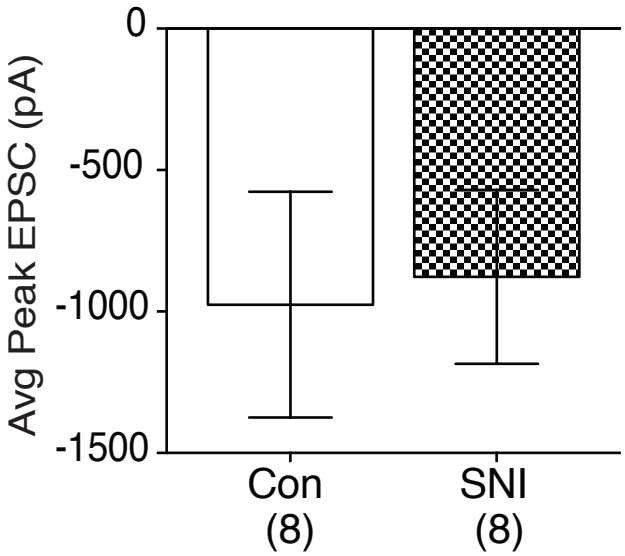

A.
Control
20 mV
500 ms
Taxol
20 mV
500 ms
SNI
20 mV
500 ms
B.
Current
250 pA
250 pA
Blue light
1.0 ms pulses
20 Hz
C.
Spikes with light ON
Spikes with light OFF
0
5
10
15
20
25
30
35
40
Control
Taxol
SNI

Figure 7: Excitation of inhibitory interneurons in layer V rACC by MD inputs is unchanged between control animals and animals with nerve injury.
Peak EPSC amplitudes recorded from fast spiking inhibitory interneurons (number of cells indicated in parentheses) in SNI (n = 2 mice) and control animals (n = 2). Error bars represent S.E.M.


Voltage clamp (-70 mV)
470 nm
light
IN
MD terminals with
ChR2-eYFP


0
-500
-1000
-1500
Avg Peak EPSC (pA)
Con
(8)
SNI
(8)

Chapter 2

The contribution of basolateral amygdala inputs to rACC-mediated pain aversion

INTRODUCTION

As is the case for the medial thalamic nuclei, the amygdala consists of a heterogenous group of nuclei that are highly interconnected. The lateral amygdala (LA) serves as the major input site for sensory information from the thalamus (LeDoux et al., 1990; Sah et al., 2003). Through extensions of the spinohypothalamic and spinothalamic pain pathways, the basolateral amygdala (BLA) receives highly processed and polymodal (including nociceptive and affective) input from the medial prefrontal cortex (mPFC), which includes the rACC, hippocampus and thalamus (midline and posterior nuclei) (LeDoux et al., 1991; McDonald 1998; Augustine 1996; Millan 1999; LeDoux 2000; Price 2000; Stefanacci and Amaral 2000). The associative processing of LA-BLA circuitry is believed to attach emotional significance to sensory stimuli (Phelps and LeDoux, 2005). This affect-related information is then transmitted to the central amygdala (CeA) (Pitkänen et al., 1997; Paré et al., 1997), as part of a well-characterized circuit underlying fear and anxiety (LeDoux 2000). The CeA also receives nociceptive input, indirectly from the parabachial nucleus, and directly from the spinal cord, via spinoamygdaloid projections (Bernard et al., 1996; Gauriau and Bernard, 2002). Because of this rich nociceptive input, the CeA has long been

termed the “nociceptive amygdala”. Through its projections to the hypothalamus and the brainstem, the CeA regulates behavioral responses to noxious stimuli.

The amygdala is thus well positioned to play an instrumental contribution to both nociceptive and affective dimensions of pain (Neugebauer et al., 2004; Veinante et al., 2013; Neugebauer and Li, 2003; Petrovic et al., 1999). In humans, imaging studies demonstrated changes in the activity of the amygdala of patients with irritable bowel syndrome (Bonaz et al., 2002), arthritis (Kulkarni et al., 2007) or mononeuropathy (Petrovic et al., 1999). Interestingly, another imaging study showed the participation of an amygdala - anterior cingulate cortex circuit in the higher subjective perception of pain in healthy subjects experiencing sadness (Yoshino et al., 2010). These studies suggest that the amygdala may be involved in the emotional aspects of acute and chronic pain. *In vivo* electrophysiological studies in rats characterized responses in the amygdala to various nociceptive stimuli (cutaneneous and visceral) and found responses that correlate with stimulus intensity and large, bilateral receptive fields (Neugebauer et al., 2004). Lesions of both CeA and BLA abolish conditioned aversion to intraplantar formalin (Tanimoto et al., 2003). Studies have also suggested that the amygdala plays an important role in forms of stress-induced analgesia and morphine-induced analgesia (Helmstetter and Bellgowan, 1992; Fox et al., 1994; McGaraughty et al., 2002; Manning and Mayer, 1995).

In our studies, we focused on the BLA, as it is densely and reciprocally connected with both the rACC and MD (Sarter and Markowitsch, 1983; McDonald 1991; Hoover and Vertes, 2007; Musil and Olson, 1988, Velayos et al., 1985). The BLA has also been heavily implicated in aversive learning, and specifically in pain-related aversion (Helmstetter and Bellgowan, 1992; LeDoux 2000). For example, similar to rACC lesions, lesions of the BLA abolish conditioned aversion to intraplantar formalin and noxious footshock (Gao et al., 2004; Tanimoto et al., 2003). Infusion of an NMDA antagonist or morphine into the BLA also reduces formalin-induced conditioned place aversion (Deyama et al., 2007). Finally, BLA lesions inhibit the chronicity of pain following peripheral nerve injury (Li et al., 2013;).

There are also cellular and molecular changes in the BLA in the setting of chronic pain – intraplantar formalin induces *c-fos* mRNA (Nakagawa et al., 2003), arthritis increases the expression of the pro-nociceptive cytokine tumor necrosis factor α (TNF-α) (Chen et al., 2013) and peripheral nerve injury increases cell proliferation in the BLA (Goncalves et al., 2008). A functional plasticity of BLA neurons is also observed in an arthritis model, namely an increase in spontaneous and evoked activity and enhanced synaptic transmission. This pain-induced plasticity can be reversed by antagonism of pathways that involve the stress-related hormone, corticotropin-releasing factor (CRF-1) (Ji et al., 2010). Consistent with these molecular changes, neutralizing TNF-α in the BLAwith antibodies reduced anxiety-like behaviors associated with arthritis (Chen et al.,

2013). CRF1 antagonism in BLA of arthritic rats also reduces pain-related vocalizations and anxiety (Ji et al., 2010).

There is also strong evidence demonstrating that the BLA-mPFC connection is important for emotional associative learning and aversive behavior related to chronic pain (Garcia et al., 1999; Moustafa et al., 2013). For example, optogenetic activation of the BLA-mPFC pathway is anxiogenic and reduces social interaction (Felix-Ortiz et al, 2015). Ji et al (2010) demonstrated that pharmacological inhibition of pain-related hyperactivity in the BLA reverses deactivation of mPFC and improves decision-making deficits in an animal model of arthritis pain. Bissiere et al (2008) found that lesions of the rACC (which projects to BLA) produce deficits in the amygdala-dependent acquisition of an association between a tone and foot shock. Malin et al (2007) found that lesions of the basolateral amygdala blocked the memory-enhancing effect of post-training intra-ACC infusions of the muscarinic cholinergic agonist oxotremorine (OXO) in an aversive learning paradigm. To our knowledge, however, the functional connectivity between the BLA and the rACC has not been directly tested or manipulated in the setting of chronic neuropathic pain. Therefore, in the present series of studies we addressed the role of the BLA-rACC pathway in pain-related aversion, using optogenetics to activate or inhibit projections from the BLA to the rACC in the setting of chronic neuropathic pain.

RESULTS

Activating BLA inputs to rACC elicits conditioned place preference

There are several models for how BLA input to the rACC might contribute to pain-related aversion. As described previously, a prevailing model is that rACC hyperactivity drives pain-related aversion – based on this, driving BLA-rACC input ought to exacerbate pain-related aversion. Other studies e.g. (Felix-Ortiz et al, 2015) suggest that BLA input to prefrontal regions is aversive and would predict a similar result. However another possibility is that input to rACC might affect pain-related aversion in more nuanced ways, depending on how that input engages specific microcircuits within rACC. To distinguish between these possibilities, we examined the consequence of activating BLA inputs to the rACC in the conditioned place paradigm, and then *in vitro*.

In these behavioral studies, we used the SNI model in mice 4 weeks after injection of AAV5-CaMKII-ChR2 virus into the BLA and implantation of optical fiber into the rACC. We measured CPP 7-10 days after SNI, allowing 5-6 weeks for viral expression (**Fig. 1A**). In marked contrast to MD input activation, optogenetic activation of BLA inputs to the rACC elicited conditioned preference (Preference for Stim Side significantly > 1), in both control animals and mice with chronic neuropathic pain, with no significant difference between the two groups (**Fig. 1B.** Difference Scores are represented in **Fig. 1–figure supplement A**). These differences between MD and BLA input activation were unexpected, and strongly suggest that BLA inputs engage distinct microcircuits within the rACC,

compared to MD input. These findings also indicate that there is not a linear relationship between input to the rACC, activity of rACC, and negative pain-related affect. Our findings, therefore, challenge existing models in which rACC hyperactivity is thought to be the main driver of pain-related aversion.

Inhibiting BLA inputs to rACC elicit the same effect as activating MD inputs to the rACC – exacerbated pain-related aversion

We next optogenetically inhibited BLA inputs to rACC to confirm that the effects we observed above reflect local activity of BLA terminals (**Fig. 2A**). Strikingly, we found that *inhibiting* BLA inputs to the rACC elicited a pattern identical to that seen when we *activated* MD inputs: aversion in animals with chronic neuropathic pain, but not in control animals (**Fig. 2B.** Difference Scores are represented in **Fig. 1–figure supplement B**). Thus, both *inhibition* of BLA inputs and *activation* of MD inputs to the rACC drive pain-related aversion. In the next chapter, we will propose a model that may explain these parallel effects.

Importantly, in a control experiment we confirmed that light delivery and virus injection alone into BLA (driving expression of eYFP only) did not lead to conditioned preference or aversion in nerve-injured animals (**Fig. 1–figure supplement C**). We conclude that the behavioral effects we observe of optogenetically activating BLA inputs to the rACC are not due to the effect of light.

Excitatory responses of layer V rACC pyramidal neurons evoked by BLA inputs are significantly increased in the setting of chronic neuropathic pain

We next wanted to study the physiological nature of the BLA-rACC connection *in vitro* and identify changes in the setting of chronic neuropathic pain that may explain the behavioral effects we observed. In striking contrast to the changes in responses of ACC neurons evoked by MD inputs, we found that EPSCs evoked by optogenetic activation of BLA inputs in animals with chronic neuropathic pain were significantly increased in both IT and SC cells compared to control animals (**Fig. 3B** and **3C**; $p < 0.05$ by unpaired t-test). This overall increase in rACC activity does not translate behaviorally into exacerbated pain-related aversion, again indicating that there is a more nuanced regulation of pain affect by distinct rACC circuits. Feedforward inhibitory responses and the excitation of inhibitory interneurons elicited by BLA inputs were very small and often not quantifiable, in both control and chronic pain conditions. These findings suggest that BLA-mediated inhibition does not simply cancel out the excitatory drive recruited by BLA inputs to the rACC. These results are also in contrast to the responses evoked by BLA inputs in the neighboring prelimbic (PL) and infralimbic (IL) regions of the mPFC, where a mix of excitatory and inhibitory responses is observed using electrical stimulation of BLA inputs (Pérez-Jaranay and Vives 1991; Ishikawa and Nakamura, 2003). Also, in contrast to MD inputs, which we found preferentially excite IT cells, BLA inputs recruit SC and IT cells equally (ANOVA using cell type, pain vs. control, and SNI vs. Taxol models as factors and including a possible interaction between cell type and condition)

DISCUSSION

In this set of studies, we find that optogenetic activation of BLA inputs to the rACC elicits conditioned preference in both control animals and animals with chronic neuropathic pain. This is the same effect we observe with *inhibiting* MD inputs. Strikingly, we find that *inhibition* of BLA inputs to the rACC elicits the same effect as *activation* of MD inputs – conditioned aversion only in animals with chronic neuropathic pain. Finally, we find that in the setting of chronic neuropathic pain, excitatory responses of layer V rACC pyramidal neurons to BLA input activation are significantly *enhanced*, whereas those evoked by MD inputs are significantly *decreased*. Taken together, these three observations strongly suggest that these two sources of inputs converge on similar downstream mechanisms (likely an rACC output neuron), but with opposite sign. In the next chapter, we will take a closer look at layer V rACC pyramidal neuron subtypes and their contribution to pain-related affect.

Involvement of BLA in appetitive circuits may explain effect on pain-related aversion

Given the prominent contribution of the BLA to aversive behavior, our finding that activating projections from the BLA to the rACC elicited conditioned place preference was unexpected. It is particularly surprising that the enhanced excitatory responses of rACC neurons to BLA input activation in the setting of chronic pain do not translate behaviorally into an increase in pain-related aversion.

Although the BLA is a key contributor to aversive learning, other studies have demonstrated its role in appetitive or reward circuits. For example, Beyeler et al (2016) demonstrated that reward and aversion are parsed into discrete circuits defined by the efferent targets of BLA neurons. Specifically, BLA neurons that project to the nucleus accumbens (NAc) responded preferentially to a conditioned stimulus signaling reward, whereas BLA neurons that project to the central amygdala (CeA) responded preferentially to the conditioned stimulus signaling an aversive outcome. In another study, Kim et al (2016) identified two genetically distinguishable pathways from the BLA to the CeA that promote or suppress appetitive behavior.

In our experiments, we are not differentiating between “aversive” and “appetitive” relevant neurons in the BLA. Presumably, the virus containing the optogenetic proteins infected both populations of neurons as well as their efferent axons. We are not aware of studies that examined whether these distinct populations of BLA neurons differentially innervate the rACC and/or neighboring regions of the mPFC. It will, therefore, be of interest for future experiments to selectively target “aversive” and “appetitive” neurons in the BLA, using the genetic markers identified by Kim et al (2016), so as to study their connectivity to rACC layer V pyramidal neurons and potential roles in pain-related aversion.

Patters of connectivity of BLA inputs on to layer V rACC pyramidal neurons

The differential targeting of BLA inputs to distinct pyramidal neurons has been demonstrated previously in the prelimbic (PL) and infralimbic (IL) regions of the mPFC. Cheriyan et al (2016) optogenetically stimulated BLA inputs to layer V and layer II/III of the PL and IL and found differential targeting of BLA to cortico-PAG (CP) and corticoamygdalar (CA) pyramidal neurons of PL and IL that also depended on laminar location of these neurons. Little and Carter (2013) showed in layer II of the mPFC, BLA projections preferentially targeted CA neurons over neurons that project to the contralateral-mPFC (equivalent to IT neurons in our study). These studies highlight the importance of describing the connectivity between brain regions on the basis of heterogeneous populations of neurons.

In these set of studies, we further expand on prior description of connectivity between BLA inputs and rACC pyramidal neurons. Specifically, we find that BLA inputs recruit SC and IT neurons equally and that this recruitment is predominantly excitatory. Furthermore, along with the results described in the previous chapter, our study provides novel evidence that BLA and MD inputs to the rACC have different patterns of connectivity onto layer V rACC pyramidal neurons. Most importantly, we found that MD inputs preferentially recruit IT neurons, while BLA inputs do not show any preferential recruitment. These findings underscore the functional heterogeneity of rACC neurons, which could not be appreciated in lesion or pharmacological studies. IT cells influence cortical processing of inputs, both locally and across the hemispheres, whereas SC cells

are positioned to exert top-down control over subcortical structures. Thus, IT and SC neurons can differentially modulate affective processing by recruiting distinct networks downstream of the rACC. These networks likely underlie the distinct behavioral effects of stimulating MD and BLA inputs in the setting of chronic neuropathic pain. This will be explored more fully in the following chapter.

The BLA and MD differentially modulate processing of pain-related aversion in the rACC

Our study is the first to elucidate the effects of manipulating any input to the rACC on pain-related aversion and the first to demonstrate that MD and BLA inputs can differentially modulate the processing of pain-related aversion in the rACC. Our results indicate that the information that these inputs transmit to the rACC is qualitatively and quantitatively different. For example, our results described in the previous chapter suggest that MD inputs transmit an aversive signal to the rACC that is enhanced in the setting of pain. In contrast, BLA inputs may be transmitting a rewarding or appetitive signal to the rACC that is enhanced in the setting of chronic pain.

Given that the rACC, BLA and MD are all reciprocally connected, it is also possible that there is an interaction between these regions that we simply cannot pick up in our experiments. For instance, MD and BLA inputs may be interacting to produce a composite percept of pain-related aversion in the rACC. One approach to address this interaction would be to use two opsins that are each

excited by distinct wavelengths of light. In testing the possibility of this approach, we injected a virus containing a red-shifted opsin called Chrimson driven by the Synapsin promoter into the MD thalamus and activated the terminals in the rACC. In order to determine whether there was cross-activation between Chrimson and Channelrhodopsin2, we shined blue light at different intensities and measured evoked responses in pyramidal neurons. To our concern, we found significant cross-activation of Chrimson by blue light, therefore these experiments could not be further developed. In the future however, it would be interesting to activate BLA and MD inputs in different spatio-temporal patterns to further elucidate the connections between these regions and the rACC and their role in pain-related aversion.

In the next chapter, we take a closer look at the layer V rACC pyramidal neurons that are differentially recruited by MD and BLA inputs, and investigate whether a particular subtype can act as a downstream effector of these inputs.

METHODS

All experiments were conducted in accordance with procedures established by the Administrative Panels on Laboratory Animal Care at the University of California, San Francisco.

Animals. All experiments were performed with male C57BL/6J mice (Simonsen Laboratory). Animals were group-housed under standard conditions with *ad libitum* access to food and water. For all electrophysiology experiments all animals were housed under a standard 12 hr light/dark cycle. For behavioral experiments conducted in red light, animals were housed under a reverse 12 hr light/dark cycle for 3 days prior to and for the duration of the experiment. Sample sizes were chosen based on effect sizes from previous experiments.

Slice preparation. We cut 250 μm coronal slices from 8 to 10-week-old male C57BL6 mice (Gee et al., 2012). Slices were cut in a chilled slicing solution in which Na+ was replaced by sucrose, then incubated in warmed artificial cerebrospinal fluid (ACSF) at 30°C–31°C for ~1 hr before being used for recordings. ACSF contained 126 mM NaCl, 26 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl, and 10 mM glucose. We secured the slice by placing a harp along the midline between the two hemispheres.

Intracellular recording. We obtained somatic whole-cell patch recordings from visually identified pyramidal cells in layer V of the rACC using differential contrast video microscopy on an upright microscope (BX51WI; Olympus). Recordings were made using a Multiclamp 700A (Molecular Devices). Except when otherwise noted, patch electrodes (tip resistance = 2–6 MOhms) were filled

with the following (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 EGTA, 2 MgCl, 2 MgATP, and 0.3 NaGTP (pH adjusted to 7.3 with KOH). All recordings were at 32.5±1°C. Series resistance was usually 10–20 MΩ, and experiments were discontinued above 30 MΩ.

Voltage clamp recordings: To record EPSCs, we held cells at -70 mV and recorded responses to BLA inputs evoked by 1.0 ms light pulses delivered at trains of 5, 10 and 20 Hz.

Expression of optogenetic proteins: We expressed channelrhodopsin (ChR2) in terminals from BLA using an AAV5 vector that contains a gene encoding ChR2-eYFP under control of the CaMKIIα promoter. We expressed archaerhodopsin from BLA terminals using an AAV5 vector that contains a gene encoding eArch3.0-eYFP under the control of the Synapsin promoter. For activation experiments, we injected a control virus containing eYFP alone under control of the CaMKII promoter. All adeno-associated viruses (AAV) were designed in the Deisseroth Lab and purchased from the UNC Vector Core. For virus injections in BLA, 0.35 µl of virus was injected at a rate of 100 nl/min and the stereotactic coordinates were: -1.34 A/P, +3.1, -4.73 D/V (in millimeters, relative to Bregma). All injections were made in 3-4 week old male C57BL6 animals, and we waited 4-6 weeks after virus injection before preparing brain slices.

***In vitro* ChR2 stimulation.** We stimulated ChR2 in BLA terminals using flashes of light generated by a Lambda DG-4 high-speed optical switch with a 300W Xenon lamp, and an excitation filter set centered around 470 nm, delivered

to the slice through a 40X objective (Olympus). Illumination was delivered across a full high-power (40X) field.

***In vivo* optogenetic stimulation.** For all behavioral experiments, we injected mice with a ChR2-eYFP or eYFP virus and implanted them with a chronic unilateral custom-made optical fiber targeted to the rACC (+1.51 mm A/P, +0.25 mm M/L, -1.13 mm D/V relative to Bregma) To habituate the mice to the tethering procedure we connected them to a 'dummy' optical patch cable 2 days before the experiment, each day for 30–60 min. Following the tethering procedure, we ran mice in the behavioral procedure (see below). For channelrhodopsin stimulation experiments, we used a 10 mW laser with a stimulation frequency of 20 Hz and 5.0 ms light pulse duration for all behavioral experiments. Total light power for all experiments was 5-10 mW. For archaerhodopsin experiments, continuous stimulation at a power of 2-2.5 mW was used.

Neuropathic pain models: Chemotherapy (Taxol)-induced neuropathy and the spared nerve injury (SNI) model were used. Four intraperitoneal injections (i.p) of Taxol (diluted in 40% DMSO) at a dose of 1mg/kg were made every other day. Control animals received saline injections. In the SNI model, the tibial branch of the sciatic nerve was isolated, and then 8-0 silk suture was used to ligate the sural and common peroneal branches of the sciatic. Next the ligated branches were transected distal to the ligature and 2-4 mm of each distal nerve stump removed. Control animals had no surgery. For all physiology experiments, the experimenter had a colleague sacrifice the animal so that experimenter was

blind to the eartag and genotype (control or chronic neuropathic pain) before slicing. For all behavior experiments, a colleague did all of the SNI surgeries and the code was broken at the time of data analysis. Each cage of mice included both experimental and control mice.

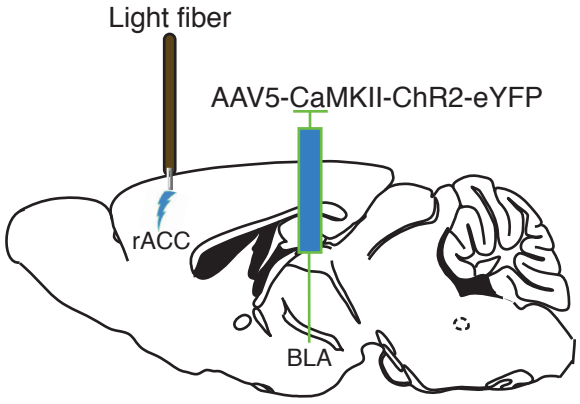
Conditioned Place Preference. The conditioned place preference (CPP) apparatus consists of a three-chambered box, a left chamber with vertical black and white stripes on the wall and a punched metal floor, a middle neutral chamber with grey walls and a smooth floor, and a right chamber with black and white spots on the wall and a mesh floor. The CPP test consists of 4 days. Day 1 is the Pre-Test Day in which mice have access to all three chambers, and we record the time spent in each chamber. On days 2 and 3, animals are restricted to either the left or right chamber (counterbalanced across all mice) and receive either 5.0 ms flashes of blue light at 20 Hz (stimulation) for 20 min, or no stimulation for 20 min. Approximately 4 hr later, the mice are restricted to the opposite chamber and receive the other treatment (stimulation or no stimulation). 24 hr after the last conditioning session on Day 4 – Test Day – the mice again have access to all three chambers, and time spent in each chamber is recorded. We calculated a Preference Score for the stimulated chamber (The ratio of the time spent in the stimulated side on Test day to Pre-Test day) and a Difference Score (The time spent in the stimulated chamber on Pre-Test day subtracted from the time spent in that chamber on Test day). Animals were not used if they spent more than 75% of total time spent in one chamber on Pre-Test day.

Statistical analysis. We used Student's t-tests (two-tailed, unpaired) to

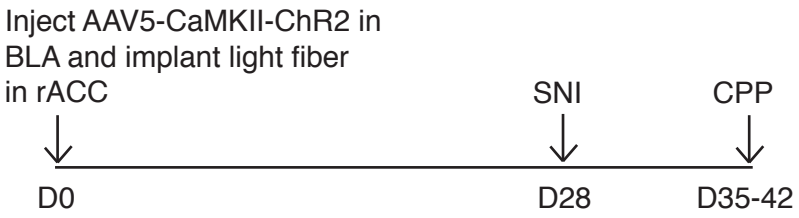
compare pairs of groups, unless there were repeated measurements or unpaired observations, in which case we used ANOVA. Automatic tests in Prism were used to confirm normality and suitability for t-tests. All of the data are represented as means with standard error of the mean. Outliers were defined as three standard deviations away from the mean.

Figure 1: Optogenetic activation of BLA inputs to rACC elicits conditioned preference in control animals and animals with chronic neuropathic pain.
A. AAV5-CaMKII ChR2-eYFP injection in BLA and optical activation of BLA terminals in rACC. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of control (n=7) and SNI (n=9) animals. Both groups have a Preference Score significantly > 1 (*, $p < 0.05$ by unpaired t-test.), indicating that both groups prefer the stimulated side. There is no difference in the Preference Score between control and SNI groups. Error bars represent S.E.M.

A.


Light fiber
AAV5-CaMKII-ChR2-eYFP
rACC
BLA

B.


Inject AAV5-CaMKII-ChR2 in
BLA and implant light fiber
in rACC
SNI
CPP
D0
D28
D35-42

C.

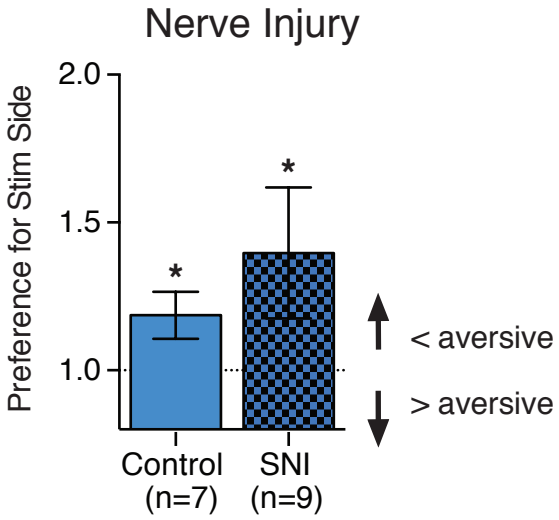
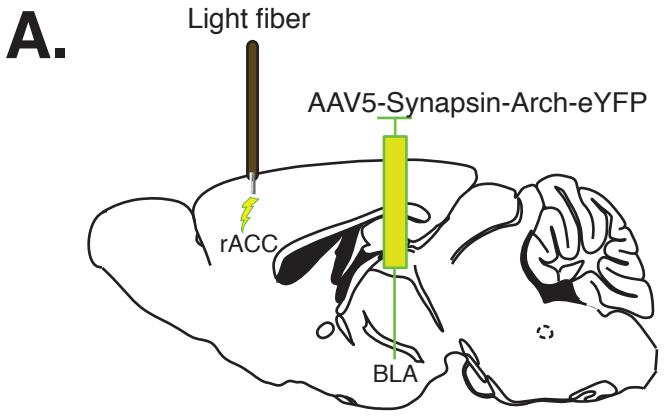

Nerve Injury
Preference for Stim Side
2.0
1.5
1.0
*
*
< aversive
> aversive
Control
(n=7)
SNI
(n=9)

Figure 2: Optogenetic inhibition of BLA inputs to rACC elicits conditioned aversion in animals with chronic neuropathic pain, identical to the effect of MD input activation.
A. AAV5-Syn-eArch-eYFP injection in BLA and optical stimulation of BLA terminals in rACC. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of control (n=9) and SNI (n=9) animals. SNI (n=9) animals have preference scores significantly < 1 (*, $p < 0.05$ by unpaired t-test.), i.e., they avoid the side paired with optogenetic inhibition of BLA inputs. Preference scores of control (n=9) animals are not significantly different from 1. Error bars represent SEM.


A.
Light fiber
AAV5-Synapsin-Arch-eYFP
rACC
BLA


B.
Inject AAV5-CaMKII-ChR2 in
BLA and implant light fiber
in rACC
SNI
CPP
D0
D28
D35-42

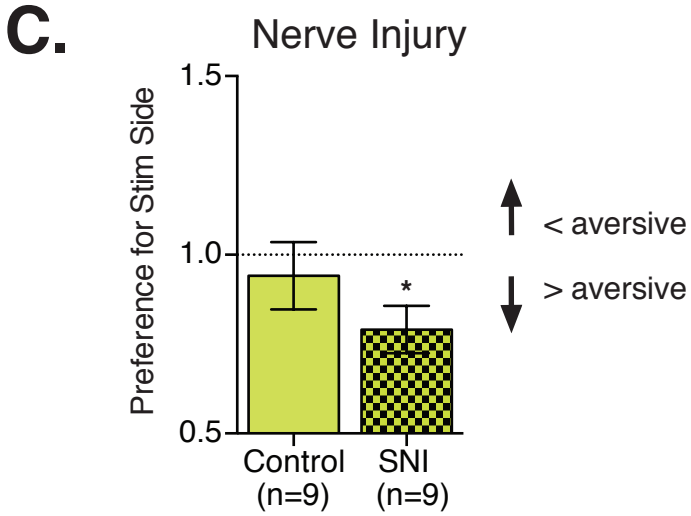
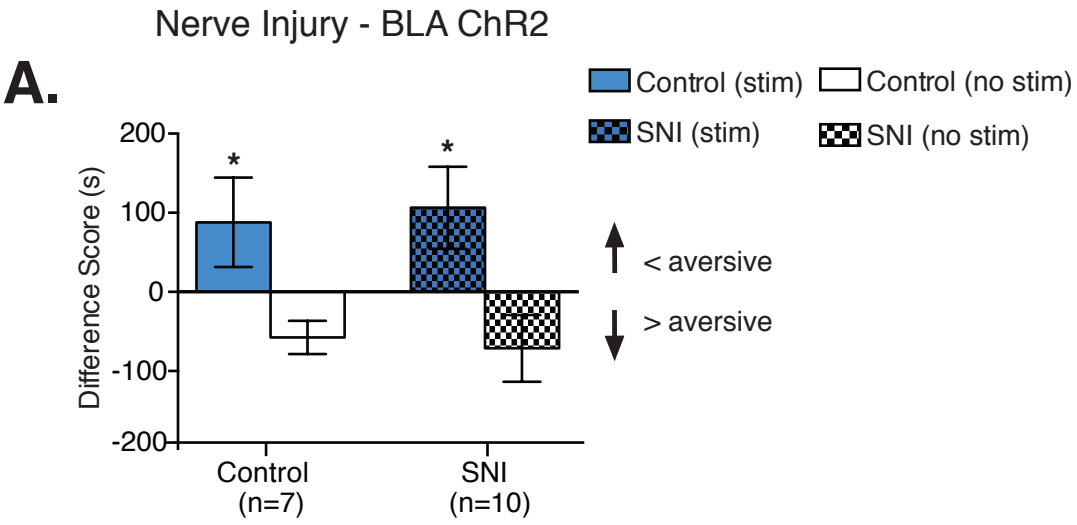
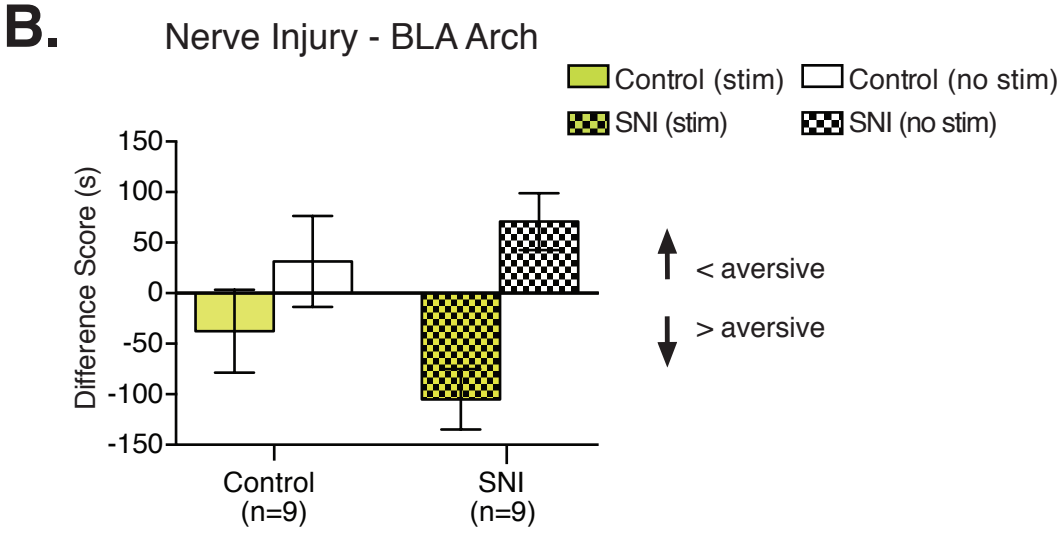

C.
Nerve Injury
Preference for Stim Side
1.5
1.0
0.5
*
< aversive
> aversive
Control
(n=9)
SNI
(n=9)

Figure 1–figure supplement: (A-B) CPP data presented as Difference Scores. (C) There is no effect of 473nm light on injured animals injected with control CaMKII-eYFP virus in BLA.

A. For animals receiving optogenetic activation of BLA inputs to the rACC, the Difference Score quantifies the difference in time (Test - Pre-test) spent in the stimulated or unstimulated chambers. A Difference Score of 0 means there was no effect of conditioning. A Difference Score < 0 means conditioning was more aversive, while a Difference Score > 0 means conditioning was less aversive. SNI and control animals have Difference Scores significantly > 0 for the stimulated side, meaning they spend more time on the side paired with optogenetic activation of BLA inputs. There is no difference in the Difference Score for the stimulated side between control and SNI animals. *, $p<0.05$ by t-test. Error bars represent S.E.M. **B.** For animals receiving optogenetic inhibition of BLA inputs to the rACC, the Difference Scores show that SNI animals spend less time on the stimulated side (Difference Score significantly > 0), while no such effect occurred in control animals. Error bars represent S.E.M. **C.** SNI animals injected with a control AAV5-CaMKII-eYFP virus in the BLA did not develop preference or aversion to the stimulated side, showing there is no effect of blue light alone. Error bars represent S.E.M.


A.
Nerve Injury - BLA ChR2
Control (stim)
Control (no stim)
SNI (stim)
SNI (no stim)
Difference Score (s)
200
100
0
-100
-200
*
*
< aversive
> aversive
Control
(n=7)
SNI
(n=10)


B.
Nerve Injury - BLA Arch
Control (stim)
Control (no stim)
SNI (stim)
SNI (no stim)
Difference Score (s)
150
100
50
0
-50
-100
-150
< aversive
> aversive
Control
(n=9)
SNI
(n=9)

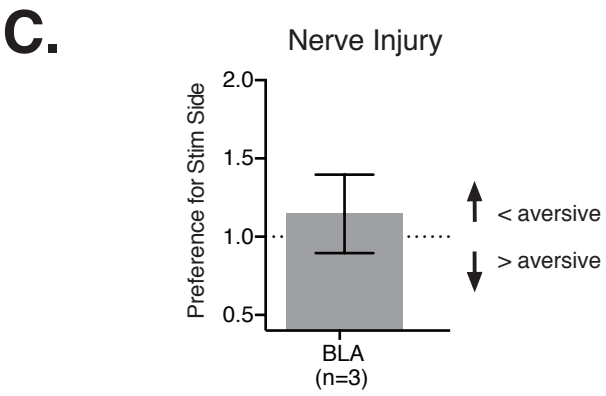
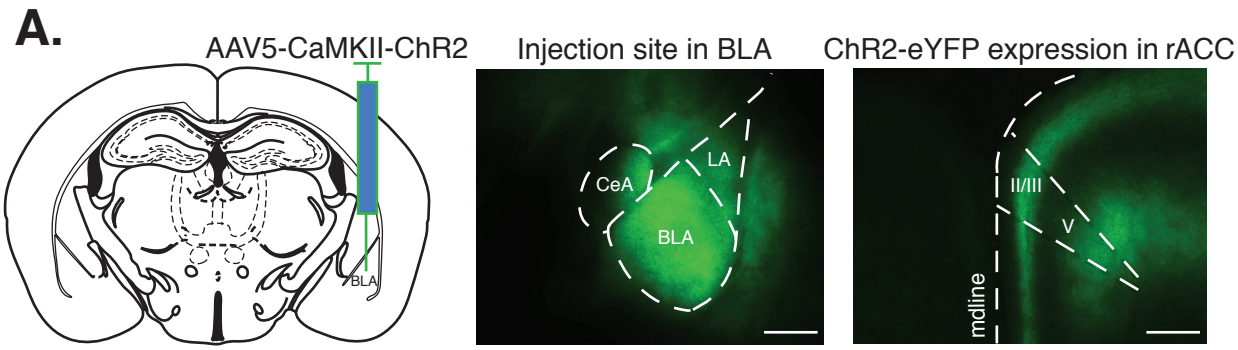
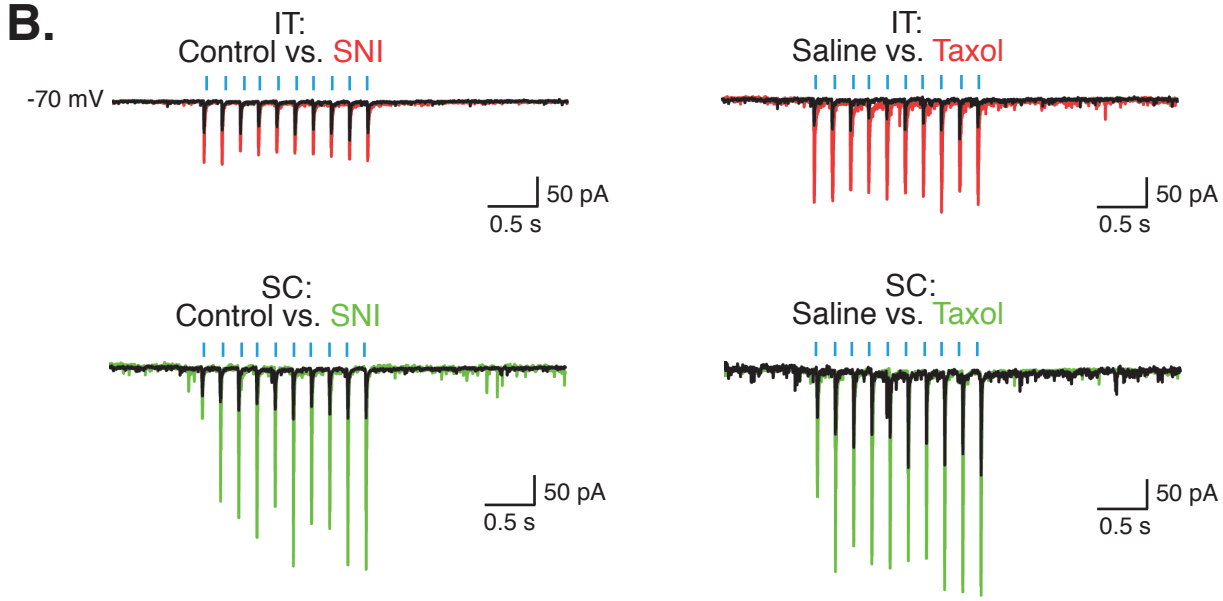
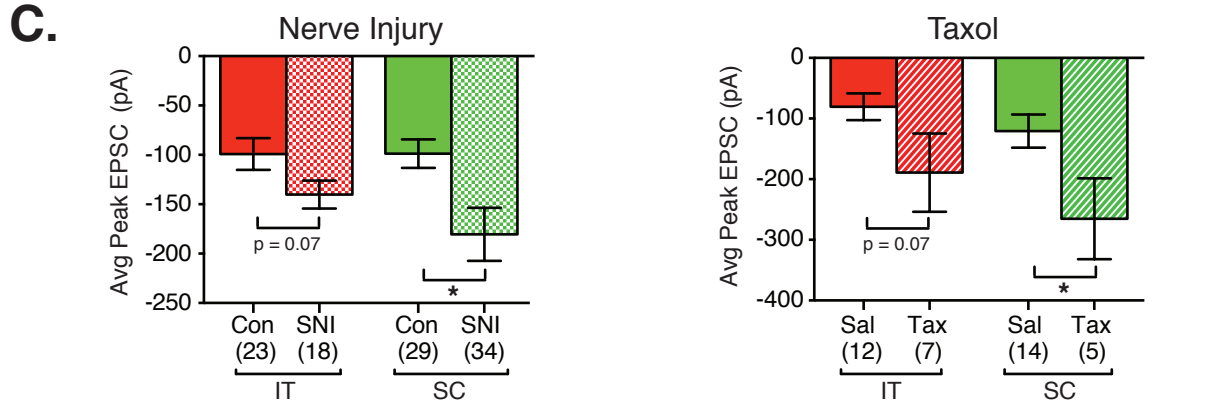

C.
Nerve Injury
Preference for Stim Side
2.0
1.5
1.0
0.5
< aversive
> aversive
BLA
(n=3)

Figure 3: Excitatory responses of layer V rACC pyramidal neurons evoked by optogenetic activation of BLA inputs are increased in SNI and Taxol animals compared to control animals.
A. (Left) Coronal section of a mouse brain at Bregma -1.34 mm showing injection of AAV5-CaMKII-ChR2-eYFP into the basolateral amygdala (BLA). (Middle) Representative low power image shows ChR2-eYFP expression within the targeted BLA. LA: lateral amygdala; CeA: central amygdala. (Right) Low power image of ChR2-eYFP expression in layer V of rACC. Scale bar = 50 μm. **B.** Population average of excitatory post-synaptic currents (EPSCs; voltage clamp responses at -70 mV) to 5.0 Hz trains of 1ms light pulses recorded from IT and SC cells in SNI (n = 4 mice) vs. control mice (n = 4) and Taxol (n = 3) vs. saline-treated mice (n = 4). **C.** The amplitude of the first EPSC in 5, 10, or 20 Hz trains is averaged to calculate the peak EPSC amplitude of the cell. Quantification of peak EPSC amplitude in IT or SC cells in SNI or Taxol animals (number of cells indicated in parentheses) compared to controls, *, $p < 0.05$ by unpaired t-test. Error bars represent S.E.M.


A.
AAV5-CaMKII-ChR2
Injection site in BLA
ChR2-eYFP expression in rACC
BLA
CeA
LA
BLA
II/III
V
mdline


B.
IT:
Control vs. SNI
-70 mV
50 pA
0.5 s
IT:
Saline vs. Taxol
50 pA
0.5 s
SC:
Control vs. SNI
50 pA
0.5 s
SC:
Saline vs. Taxol
50 pA
0.5 s


C.
Nerve Injury
Avg Peak EPSC (pA)
0
-50
-100
-150
-200
-250
p = 0.07
*
Con (23)
SNI (18)
Con (29)
SNI (34)
IT
SC
Taxol
Avg Peak EPSC (pA)
0
-100
-200
-300
-400
p = 0.07
*
Sal (12)
Tax (7)
Sal (14)
Tax (5)
IT
SC

Chapter 3

The contribution of distinct rACC output neurons to pain aversion

INTRODUCTION

Is there a simple way to understand and unify the physiological and behavioral changes described in the previous chapters and posit a model that explains convergent effects of manipulating distinct rACC inputs – that *activating* MD inputs and *inhibiting* BLA inputs both elicited aversion specifically in animals with chronic pain? We find that in animals with pain, the ratio of excitatory to inhibitory currents elicited by MD inputs becomes predominately inhibitory, specifically in SC neurons of the rACC. By contrast, the ability of BLA inputs to excite both SC and IT neurons of the rACC is enhanced in the setting of chronic pain. This observation suggests that in animals with pain, activating MD and BLA inputs elicit opposing effects on SC neuron excitability. Taking these findings together, we propose the following model to explain the similar pattern of effects of activating MD inputs and inhibiting BLA inputs on pain-related aversion. Specifically, in animals with pain, activating MD inputs and inhibiting BLA inputs may both elicit a net reduction in SC neuron activity, by increasing feedforward inhibition and suppressing excitatory input, respectively (**Fig. 1A**). Conceivably, reduced SC neuron activity in animals with chronic pain could be a common link that drives pain-related aversion in response to both of these manipulations (**Fig. 1B**)**.** If this model is correct, then direct inhibition of SC neurons should also lead to pain-related aversion (**Fig. 1B).**

RESULTS

Inhibiting SC neurons in rACC exacerbates pain-related aversion

To test this model, we measured the effects of inhibiting SC neurons within the rACC in mice with chronic neuropathic pain and in control animals in the conditioned place paradigm. In these experiments, we injected canine adenovirus that encodes Cre (CAV2-Cre virus) into the MD thalamus. This virus is taken up by axon terminals and traffics retrogradely to the rACC, where we injected a eYFP-tagged virus that drives Cre-dependent expression of the inhibitory opsin Arch (**Fig. 2A and C)**. We confirmed that SC neurons in the infragranular layers of rACC were labeled by eYFP (**Fig. 2B)**. Consistent with the predictions of our model, animals with chronic neuropathic pain (Taxol model) spend less time on the side that was paired with optogenetic inhibition of SC neurons, while there is no significant effect in control animals (**Fig. 2D)**. In other words, inhibiting SC neurons exacerbates pain-related aversion.

Evidence for differential regulation of pain-related aversion by distinct layer V rACC output neurons

In an important control experiment, we also studied the effect of inhibiting IT neurons, by injecting the CAV2-Cre virus in contralateral rACC and Cre-dependent Arch in the side of the light implant. We found that both control animals and animals with chronic neuropathic pain showed neither a preference nor aversion to the side paired with inhibition of IT neurons (**Figure 3**). This finding is the first to describe differential roles of distinct populations of rACC

neurons and suggests that it is the SC subpopulation of neurons that contributes to pain-related aversion.

Activation of layer V rACC pyramidal neuron subtypes has no effect on pain-related aversion

In the previous set of experiments we show that inhibition of SC neurons, but not IT neurons exacerbates pain-related aversion. We next wanted to observe whether direct activation of SC or IT neurons exerts similar divergent effects on pain-related aversion. Specifically, our model would predict that activation of SC neurons would elicit preference in animals with chronic neuropathic pain. We used the same approach outlined above and injected the CAV2-Cre virus in either MD thalamus or the contralateral cortex and a Cre-dependent virus in rACC to drive expression of the excitatory opsin ChR2 within either SC neurons or IT neurons, respectively. There was a non-significant trend towards preference with SC neuron activation in animals with chronic neuropathic pain (**Figure 4)**. There was no effect of activation of IT neurons in either animals with chronic pain or control animals (**Figure 5)**.

Targeting outputs of layer V rACC pyramidal neuron subtypes does not have an effect on pain-related aversion

Direct activation of SC or IT neurons will affect multiple downstream targets, and perhaps could explain why we observed only a trend towards preference elicited by activation of SC neurons in animals with chronic pain. Thus, in these set of

experiments, we activated known targets of SC or IT neurons – namely the mediodorsal thalamus (MD) or contralateral cortex respectively. To do this, we injected AAV5-CaMKII-ChR2 in the rACC and implanted the light fiber in either mediodorsal thalamus or contralateral cortex. There was no effect of activation of rACC terminals in either MD (**Figure 6**) or contralateral cortex, in either control or animals with chronic neuropathic pain (**Figure 7)**. This raises two possibilities. The MD may not be the target of SC neurons that is relevant to the regulation of pain-related aversion (we discuss this possibility further below). Alternatively, activating these terminals may cause non-physiological patterns of activity that are relatively ineffective at eliciting behavioral effects, whereas inhibiting the cell bodies and suppressing endogenous patterns of activity may be more effective.

DISCUSSION

In these set of studies, we found a differential regulation of pain-related aversion by distinct populations of rACC layer V pyramidal neurons. Specifically, inhibition of SC neurons elicited aversion in animals with chronic pain. In contrast, inhibition of IT neurons had no effect on pain-related aversion. These findings not only underscore the heterogeneity of intrinsic rACC circuits, but also build on our findings from previous chapters that there are distinct input-output pathways through the rACC that mediate pain-related aversion. Importantly, these results provide further evidence that SC output neurons of the rACC may be a potential node of convergence of information from MD and BLA inputs. Specifically, we propose a model in which activating MD and inhibiting BLA inputs elicit aversion in animals with pain by suppressing activity of SC neurons. This model differs considerably from prevailing ones in which rACC hyperactivity is the main factor underlying pain-related aversion.

Novel evidence for the contribution of distinct rACC output neurons to pain-related aversion

As mentioned in previous chapters, lesion studies or non-specific activation of the rACC does not allow for the investigation of heterogeneity of rACC cells or circuits. Pharmacological manipulations have underscored rACC heterogeneity in terms of neurotransmitter systems. For example, it was shown that antagonism of NMDA receptors and not AMPA receptors in the rACC preferentially eliminated formalin-induced conditioned place aversion (Lei et al., 2004). Another study

demonstrated that $GABA_A$ but not $GABA_B$ receptors in the rACC selectively modulate pain-related aversion (LaGraize et al., 2007).

Recently, there have been some efforts to use pharmacogenetic and optogenetic tools to activate distinct populations of cortical neurons and assess the effects on pain perception. For example, Kang et al (2015) used optogenetic techniques to selectively modulate excitatory pyramidal neurons and inhibitory interneurons in the ACC and found bidirectional effects on peripheral mechanical hypersensitivity in mice. However, they only tested acute pain behaviors and not pain-related aversion as measured by a conditioned place paradigm. Importantly, they also targeted the posterior half of the ACC and not the rACC (Kang et al., 2015). Another study targeted projections from the mPFC to the nucleus accumbens and found that activation of this projection elicits conditioned preference in animals with chronic neuropathic pain (i.e. it is pain-relieving). However this study preferentially targeted the prelimbic region (PL) of the mPFC. Another recent study targeted specific populations of layer V inhibitory interneurons in the somatosensory cortex (S1) and found that pharmacogenetic inhibition of somatostatin-expressing inhibitory interneurons reversed mechanical allodynia in a mouse model of neuropathic pain. While these studies underscore the importance of cortical heterogeneity, our study is the first to address the role of distinct populations of rACC pyramidal neurons in pain-related aversion.

Potential targets of SC neurons that may be implicated in pain-related aversion

Based on other studies in the lab, we know that the SC neurons project to a number of subcortical structures, including the MD, BLA, nucleus accumbens and brainstem. Although we still do not have a complete understanding of the downstream mechanism through which SC neuron inhibition leads to pain-related aversion, at least two possibilities should be considered. One proposal arises from the fact that a population of SC neurons in the rACC likely sends excitatory projections to the periaqueductal grey (PAG), a region strongly implicated in mechanisms of pain relief (Young et al., 1997; Fardin et al., 1987). Descending projections from the PAG to the rostral ventromedial medulla (RVM) and dorsal horn of the spinal cord have been well characterized in a variety of species (Shah and Dostrovsky, 1980; Sandkuhler et al., 1987; Mantyh 1983). The PAG-RVM-spinal cord pathway comprises an essential neural circuit for opioid-based pain relief (Basbaum and Fields, 1978; Basbaum and Fields, 1984). Intra-PAG administration of the mu opioid receptor (MOR) agonist morphine, the most commonly prescribed opiate for persistent pain relief, produces naloxone-reversible analgesia (Yaksh and Rudy, 1978). Similarly, lesions of the PAG or intra-PAG administration of MOR antagonists attenuate the antinociceptive effects of systemic morphine (Wilcox et al., 1979; Ma and Han, 1991). Thus, the PAG presents itself as a good candidate for a downstream target of SC neurons that is involved in pain relief. In the setting of chronic pain, increased inhibition of SC-PAG projecting neurons could suppress excitatory drive to the PAG, thereby

reducing its antinociceptive actions and in turn exacerbate the aversiveness of a chronic pain state.

Alternatively, collaterals of SC projection neurons may drive feedforward inhibition via GABAergic interneurons on to a target that is involved in pain aversion or aversive behavior. Then suppressing the activity of SC neurons could lead to disinhibition of this target and exacerbate the aversiveness related to a chronic pain state. A good candidate is the lateral habenula (LHb), a structure that is heavily involved in the processing of aversive information (Matsumoto and Hikosaka, 2009; Hikosaka, 2010). The spinal cord sends nociceptive information to the Lhb directly (Craig, 2003) or indirectly via the lateral hypothalamus (Dafny et al., 1996). The LHb is also closely connected with regions involved in affective processing, like the medial prefrontal cortex and insular cortex (Greatrex and Phillipson, 1982; Kim and Lee, 2012). cFos immunoreactivity in LHb was shown to increase in rats receiving a painful stimulus (Lehner et al., 2004; Shelton et al., 2012; Li et al., 2016). It has also been shown that an aversive stimulus increases the LHb input on to GABAergic interneurons in the rostromedial tegmental nucleus (RMTg) leading to a decrease in dopamine output from the ventral tegmental area (VTA) and driving aversive learning (Ji and Shepard, 2007; Jhou et al., 2009; Lavezzi and Hahns, 2011; Stamatakis and Stuber, 2012). It is also reported that morphine can produce analgesic effects by inhibiting activity of LHb neurons (Ma et al., 1992; Margolis and Fields, 2016). While a direct role for the lateral habenula in pain-related aversion is still unclear, it is possible that SC

neurons in the rACC could exert a top-down disinhibition of LHb activity in the setting of chronic pain, thereby modulating the percept of pain aversion.

The possibilities described above make the assumption that the output of SC neurons of the rACC is glutamatergic. A recent study from the lab also described long-range, GABAergic projections from the prelimbic (PL) and infralimbic (IL) regions of the mPFC (Lee et al., 2014). These GABAergic projections terminate in a number of subcortical regions, including the basolateral amygdala (BLA), claustrum and the striatum. In fact, it was found that optogenetically stimulating these long-range GABAergic projections from the mPFC to the nucleus accumbens elicits avoidance behavior in a real-time place preference task; thus, these projections are thought to encode an aversive signal. Such long-range GABAergic projections originating from the rACC have not yet been found or described, but the possibility of GABAergic output from SC neurons adds further complexity to potential downstream circuits involved in pain-related aversion.

METHODS

All experiments were conducted in accordance with procedures established by the Administrative Panels on Laboratory Animal Care at the University of California, San Francisco.

Animals. All experiments were performed with male C57BL/6J mice (Simonsen Laboratory). Animals were group-housed under standard conditions with *ad libitum* access to food and water. For behavioral experiments conducted in red light, animals were housed under a reverse 12 hr light/dark cycle for 3 days prior to and for the duration of the experiment. Sample sizes were chosen based on effect sizes from previous experiments.

Expression of optogenetic proteins: We expressed channelrhodopsin (ChR2) in terminals from rACC using an AAV5 vector that contains a gene encoding ChR2-eYFP under control of the CaMKIIα promoter. All adeno-associated viruses (AAV) were designed in the Deisseroth Lab and purchased from UNC Vector Core. For injections into the rACC, 0.5-0.7 µl of virus was injected at a rate of 100 nl/min. Coordinates for the rACC (in millimeters relative to Bregma) were +1.50 A/P, +0.26 M/L, -1.50 and -1.75 D/V to ensure spread of virus in the ACC. To label subcortically projecting (SC) neurons or intratelecephalic (IT) neurons, we used the CAV2-Cre virus from BioCampus. For virus injections in MD, 0.35 µl of virus was injected at a rate of 100 nl/min. Coordinates for MD thalamus were (in millimeters relative to Bregma): -1.7 A/P, +0.3 M/L, and -3.43 D/V. For injections into the contralateral rACC, coordinates were (in millimeters relative to Bregma): +1.50 A/P, -0.26 M/L, -1.50 and -1.75

D/V. All injections were made in 3-4 week old male C57BL6 animals, and we waited 4-6 weeks after virus injection before preparing brain slices.

***In vivo* optogenetic stimulation.** For stimulation of rACC terminals in MD thalamus or contralateral cortex, chronic unilateral optical fibers were implanted into MD at -1.7 A/P, +0.3 M/L, and -2.95 D/V or into contralateral rACC at +1.51 A/P, -0.25 M/L, -1.13 D/V (in millimeters relative to Bregma). For activation of SC or IT neurons, an optical fiber was targeted to the rACC (+1.51 mm A/P, +0.25 mm M/L, -1.13 mm D/V relative to Bregma). We connected mice to a 'dummy' optical patch cable 2 days before the experiment each day for 30–60 min to habituate them to the tethering procedure. Following the tethering procedure, we ran mice in the behavioral procedure (see below). For channelrhodopsin stimulation experiments, we used a 10 mW laser with a stimulation frequency of 20 Hz and a 5.0 ms light pulse duration for all behavioral experiments. Total light power for all experiments was 5-10 mW. For archaerhodopsin experiments, continuous stimulation at a power of 2-2.5 mW was used.

Neuropathic pain models: For these experiments, the chemotherapy (Taxol)-induced neuropathy was used. Four intraperitoneal injections (i.p) of Taxol (diluted in 40% DMSO) at a dose of 1mg/kg were made every other day. Control animals received saline injections. For all behavior experiments, a colleague did all of the Taxol injections respectively and the code was broken at the time of data analysis. Each cage of mice included both experimental and control mice.

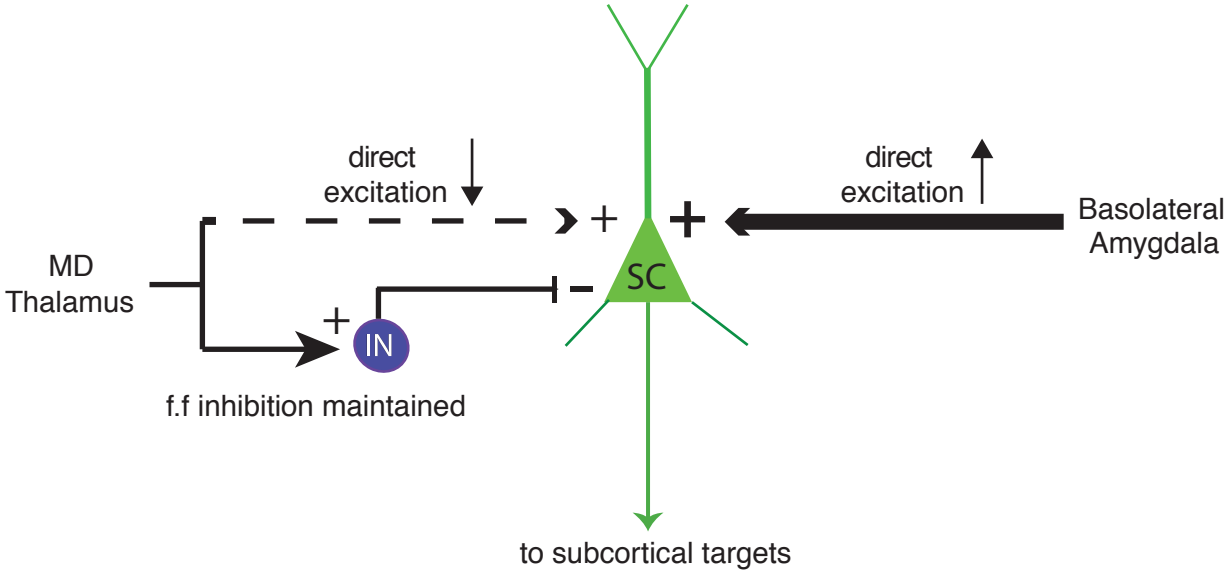
Conditioned Place Preference. The conditioned place preference (CPP)

apparatus consists of a three-chambered box, a left chamber with vertical black and white stripes on the wall and a punched metal floor, a middle neutral chamber with grey walls and a smooth floor, and a right chamber with black and white spots on the wall and a mesh floor. The CPP test consists of 4 days. Day 1 is the Pre-Test Day in which mice have access to all three chambers, and we record the time spent in each chamber. On days 2 and 3, animals are restricted to either the left or right chamber (counterbalanced across all mice) and receive either 5.0 ms flashes of blue light at 20 Hz (stimulation) for 20 min, or no stimulation for 20 min. Approximately 4 hr later, the mice are restricted to the opposite chamber and receive the other treatment (stimulation or no stimulation). 24 hr after the last conditioning session on Day 4 – Test Day – the mice again have access to all three chambers, and time spent in each chamber is recorded. We calculated a Preference Score for the Stimulated Side (The ratio of the time spent in the stimulated side on Test day to Pre-Test day) and a Difference Score (The time spent in the stimulated chamber on Pre-Test day subtracted from the time spent in that chamber on Test day). Animals were not used if they spent more than 75% of total time spent in one chamber on Pre-Test day.

Statistical analysis. We used Student's t-tests (two-tailed, unpaired) to compare pairs of groups, unless there were repeated measurements or unpaired observations, in which case we used ANOVA. Automatic tests in Prism were used to confirm normality and suitability for t-tests. All of the data are represented as means with standard error of the mean. Outliers were defined as three standard deviations away from the mean.

Figure 1: Pain-related aversion elicited by MD input activation and BLA input inhibition is driven by reduction in SC neuron activity.
A. In the setting of chronic neuropathic pain, direct excitation of SC neurons evoked by MD inputs is significantly reduced, compared to control conditions (indicated by dashed arrow and smaller '+'), whereas feedforward (f.f) inhibition is maintained (indicated by solid line and '–' symbol). On the other hand, direct excitation of SC neurons evoked by BLA inputs is significantly increased in the setting of chronic neuropathic pain compared to control conditions (indicated by thicker arrow and larger '+'). Feedforward inhibition from BLA inputs is negligible.
B. Through mechanisms in **A.** we can explain the behavioral effects of optogenetic manipulations of MD and BLA inputs. In the setting of chronic neuropathic pain, optogenetically activating ('opto stim', blue arrow) MD inputs to rACC results in a shift of the E/I ratio of SC neurons towards inhibition ('net inhibition'). Since excitatory responses of SC neurons to BLA input activation increase in the setting of chronic neuropathic pain, optogenetically inhibiting ('opto inhibit', yellow arrow) BLA inputs to rACC ostensibly reduces the excitation ('reduced excitation') of SC neurons. These two manipulations, along with direct optogenetic inhibition ('opto inhibition', yellow arrow) of SC neurons would all be expected to result in a decrease in SC neuron activity, which then drives pain-related aversion.

A. Physiologial changes associated with chronic pain relative to baseline:



B. Behavioral consequence of reducing SC activity: pain-related aversion:

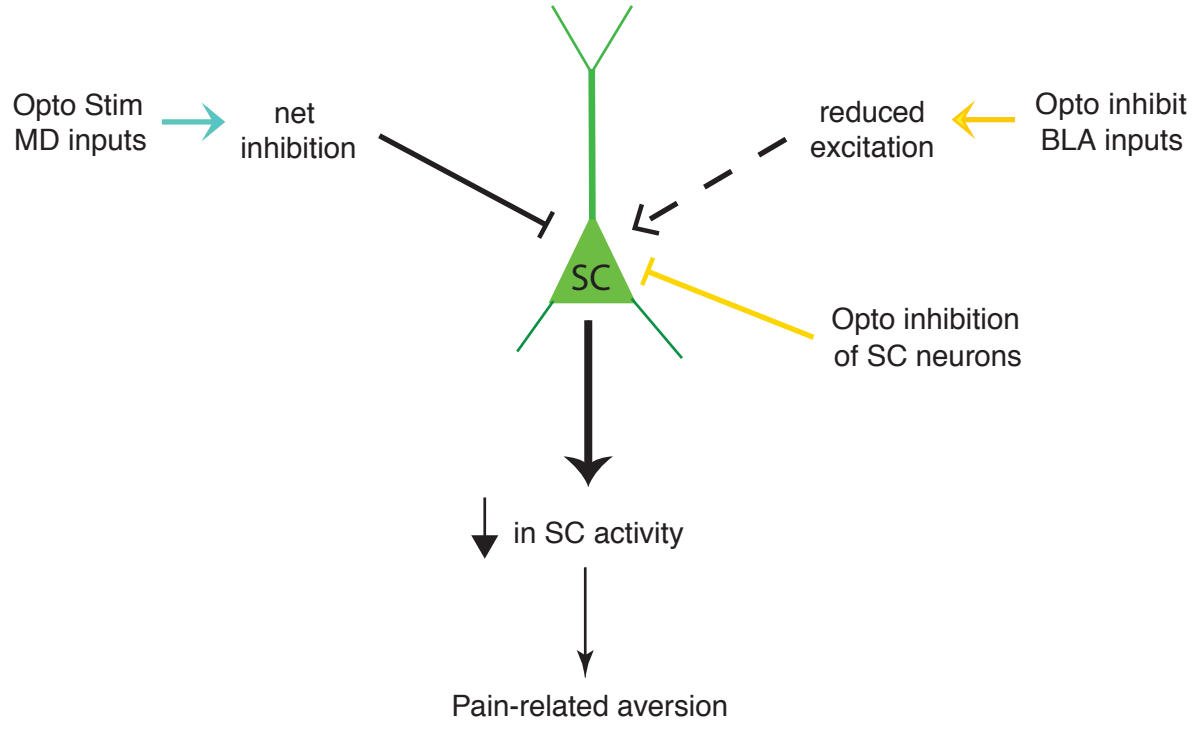


Figure 2: Optogenetic inhibition of SC neurons of the rACC elicits aversion in animals with chronic neuropathic pain.
A. Injection of CAV2-Cre in mediodorsal thalamus (MD) and AAV5-DIO-eArch-eYFP into rACC, and implantation of a light fiber over rACC, in order to target SC neurons within rACC. **B.** Sparse labeling of SC neurons by eYFP expression in superficial and deep layer V, layer VI of rACC. **C.** Timeline of injection, implantation and behavior. **D.** Quantification of Preference for Stim Side of saline injected (n=6) and Taxol injected (n=10) animals. For Taxol animals, the Preference Score for the side paired with optogenetic inhibition of SC neurons is significantly < 1 (*, $p < 0.05$ by unpaired t-test.), indicating that inhibition of SC neurons elicits aversion in animals with chronic neuropathic pain. Control animals neither prefer nor avoid the side paired with optogenetic inhibition of SC neurons. Error bars represent S.E.M.

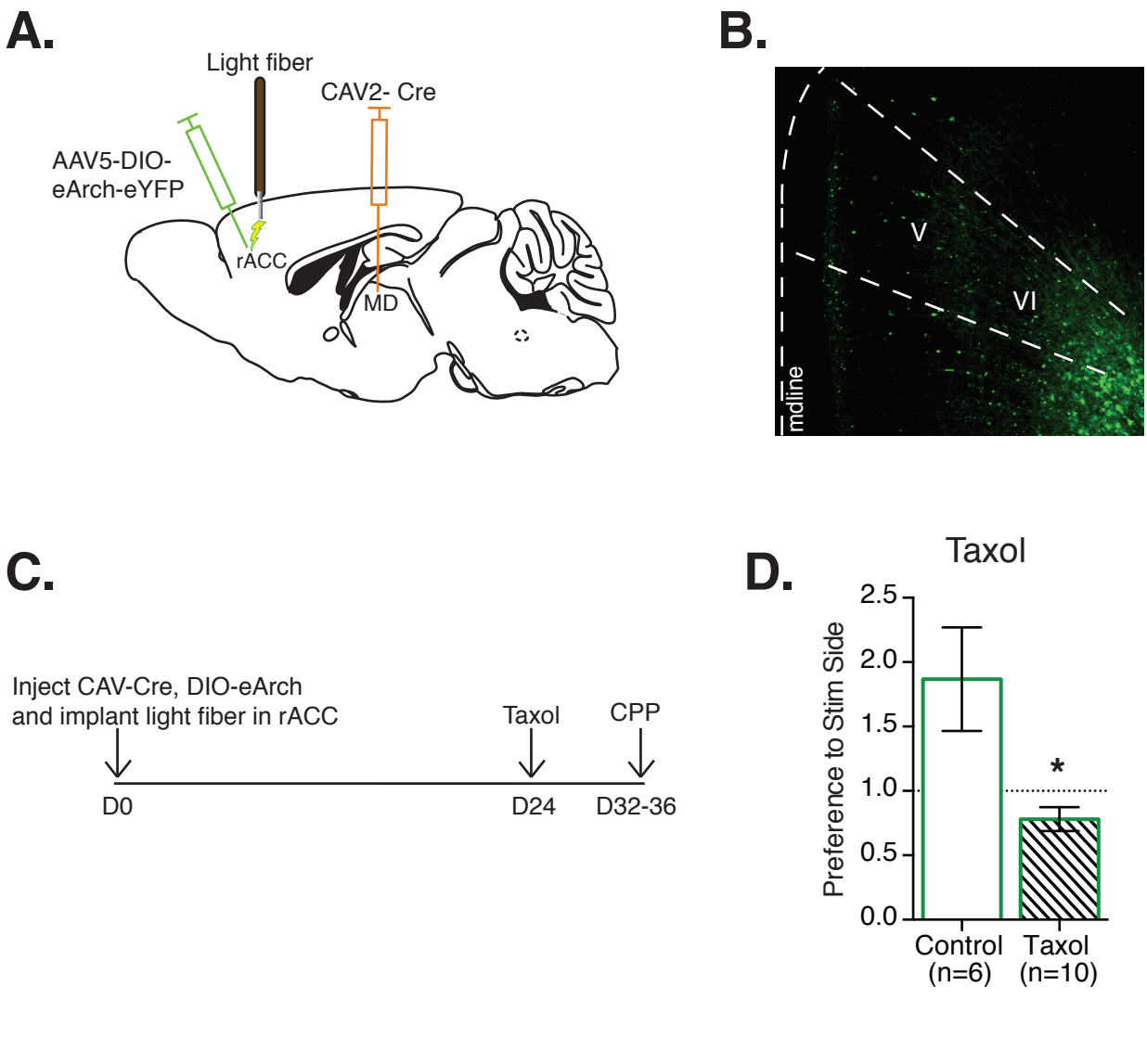
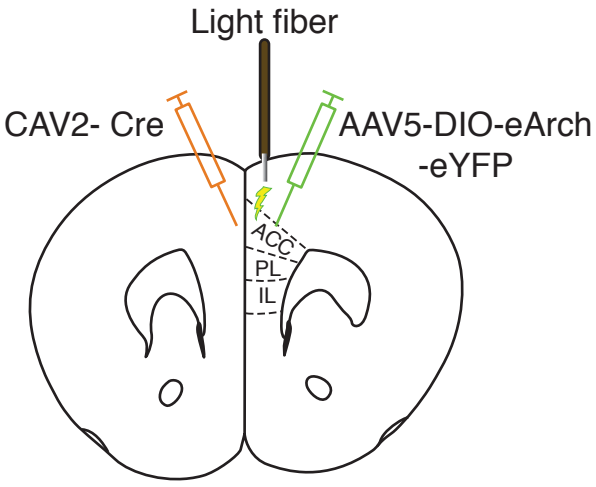
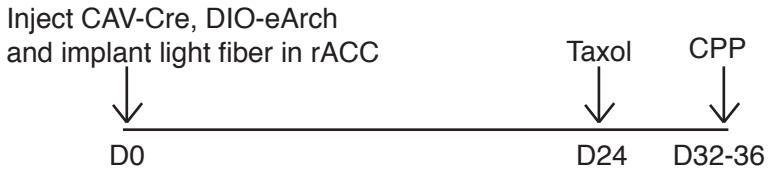

A.
Light fiber
CAV2- Cre
AAV5-DIO-
eArch-eYFP
rACC
MD
B.
V
VI
mdline
C.
Inject CAV-Cre, DIO-eArch
and implant light fiber in rACC
Taxol
CPP
D0
D24
D32-36
D.
Taxol
Preference to Stim Side
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
(n=6)
Taxol
(n=10)

Figure 3: Optogenetic inhibition of IT neurons of the rACC elicits neither aversion nor preference in pain or control animals.
A. Injection of CAV2-Cre in contralateral rACC and AAV5-DIO-eArch-eYFP and implantation of a light fiber over ipsilateral rACC, in order to target IT neurons within rACC. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of control (n = 7) and Taxol (n = 8) animals. Optogenetic inhibition of IT neurons elicits neither preference nor aversion in either controls or animals with chronic neuropathic pain (*, $p < 0.05$ by unpaired t-test.) Errors represent S.E.M.

A.


Light fiber
CAV2- Cre
AAV5-DIO-eArch
-eYFP
ACC
PL
IL

B.


Inject CAV-Cre, DIO-eArch
and implant light fiber in rACC
Taxol
CPP
D0
D24
D32-36

C.

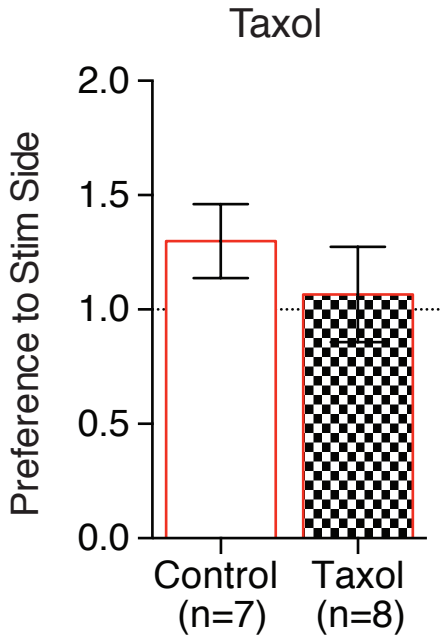
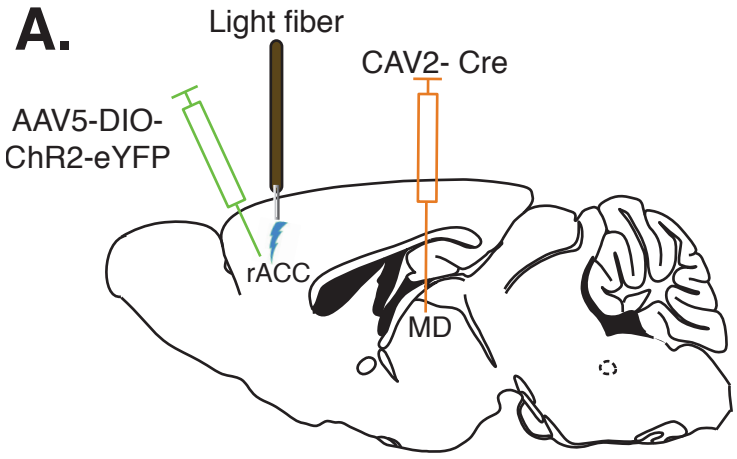
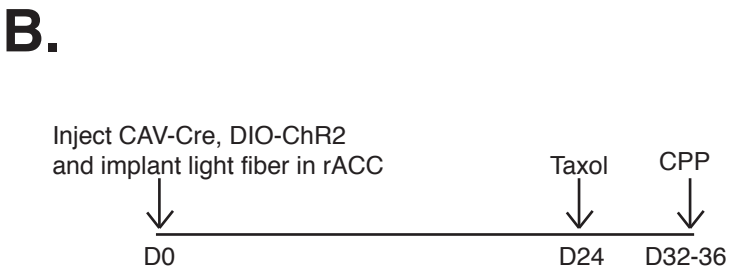

Taxol
Preference to Stim Side
2.0
1.5
1.0
0.5
0.0
Control
(n=7)
Taxol
(n=8)

Figure 4: Optogenetic activation of SC neurons in the rACC elicits only a trend of preference in animals with chronic neuropathic pain.
A. Injection of CAV2-Cre in mediodorsal thalamus (MD) and AAV5-DIO-ChR2-eYFP into rACC, and implantation of a light fiber over rACC, in order to target SC neurons within rACC. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of saline injected (n=8) and Taxol injected (n=8) animals. For Taxol animals the preference scores for the side paired with optogenetic activation of SC neurons does not elicit either aversion or preference in control animals, and a trend towards preference in animals with chronic neuropathic pain. Error bars represent S.E.M.


A.
Light fiber
CAV2- Cre
AAV5-DIO-
ChR2-eYFP
rACC
MD


B.
Inject CAV-Cre, DIO-ChR2
and implant light fiber in rACC
Taxol
CPP
D0
D24
D32-36

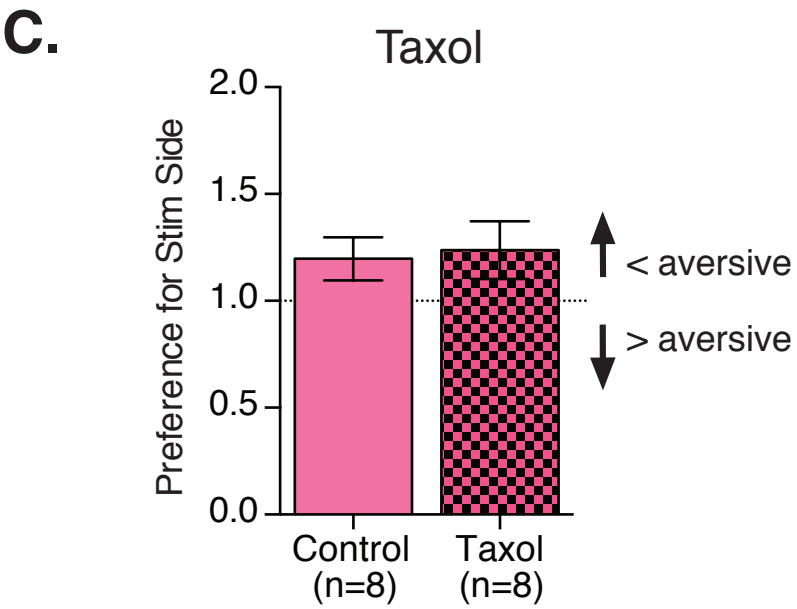

C.
Taxol
Preference for Stim Side
2.0
1.5
1.0
0.5
0.0
< aversive
> aversive
Control
(n=8)
Taxol
(n=8)

Figure 5: Optogenetic activation of IT neurons in the rACC elicits neither preference nor aversion in either controls or animals with chronic neuropathic pain.
A. Injection of CAV2-Cre in contralateral rACC and AAV5-DIO-eArch-eYFP and implantation of a light fiber over ipsilateral rACC, in order to target IT neurons within rACC. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of control (n = 8) and Taxol (n = 8) animals. Optogenetic activation of IT neurons elicits neither aversion nor preference in either control or animals with chronic neuropathic pain (*, $p < 0.05$ by unpaired t-test.) Errors represent S.E.M.

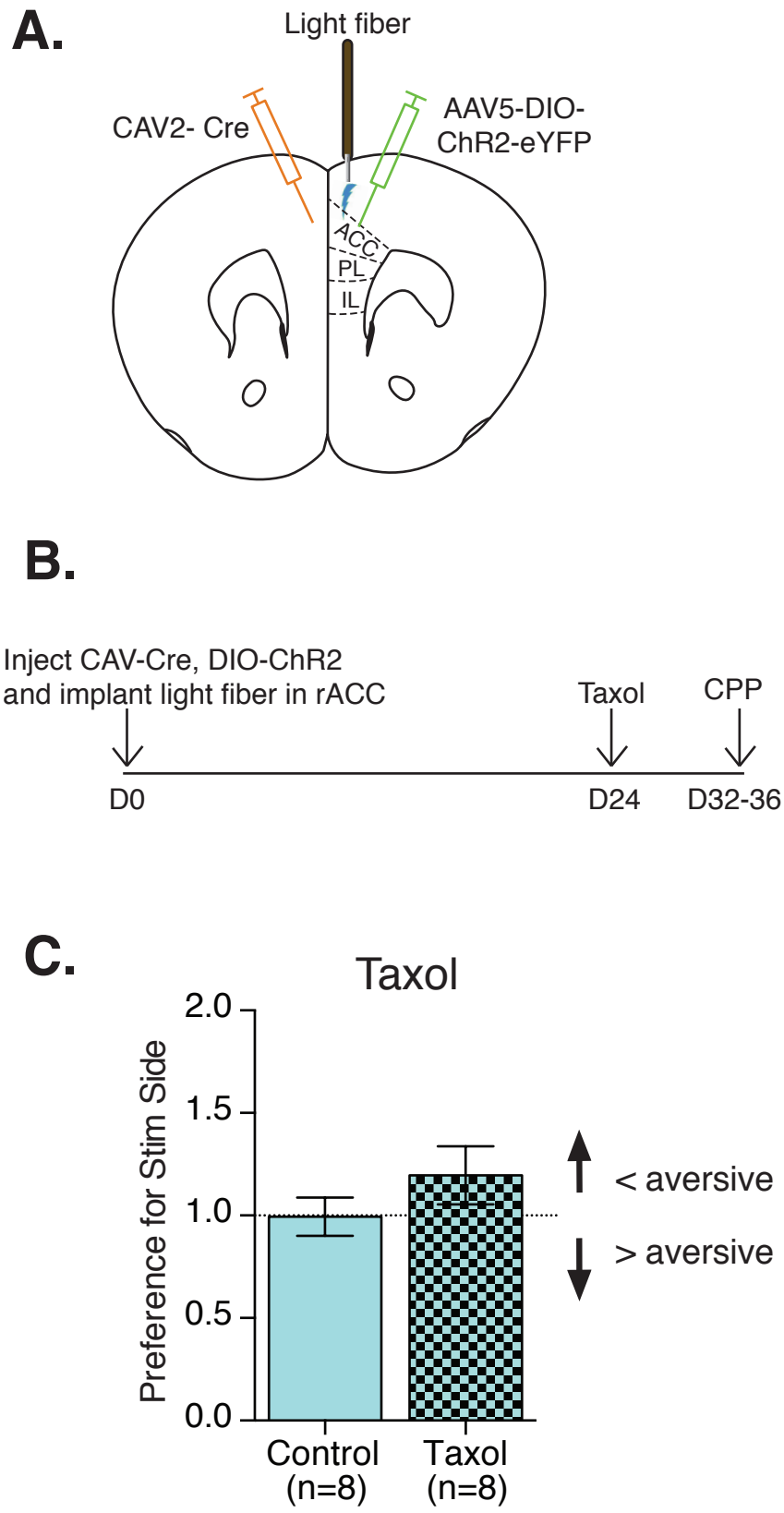
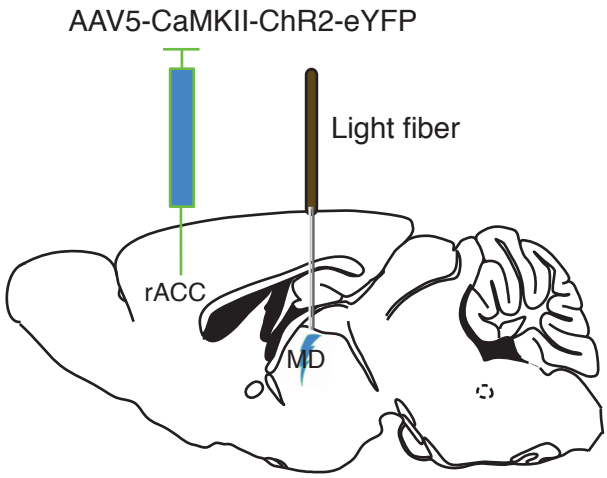
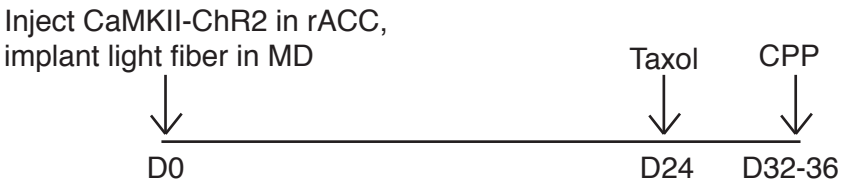

A.
Light fiber
CAV2- Cre
AAV5-DIO-
ChR2-eYFP
ACC
PL
IL
B.
Inject CAV-Cre, DIO-ChR2
and implant light fiber in rACC
Taxol
CPP
D0
D24
D32-36
C.
Taxol
Preference for Stim Side
2.0
1.5
1.0
0.5
0.0
< aversive
> aversive
Control
(n=8)
Taxol
(n=8)

Figure 6: Optogenetic activation of rACC terminals in MD elicits neither preference nor aversion in either controls or animals with chronic neuropathic pain.
A. Injection of AAV5-CaMKII-ChR2-eYFP in rACC and implantation of a light fiber in ipsilateral mediodorsal thalamus (MD), in order to target rACC terminals in MD. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of control (n = 6) and Taxol (n = 9) animals. Optogenetic activation of rACC terminals in MD is neither aversive nor preference-inducing in either control or animals with chronic neuropathic pain. (*, $p < 0.05$ by unpaired t-test.) Errors represent S.E.M.

A.


AAV5-CaMKII-ChR2-eYFP
Light fiber
rACC
MD

B.


Inject CaMKII-ChR2 in rACC,
implant light fiber in MD
Taxol
CPP
D0
D24
D32-36

C.

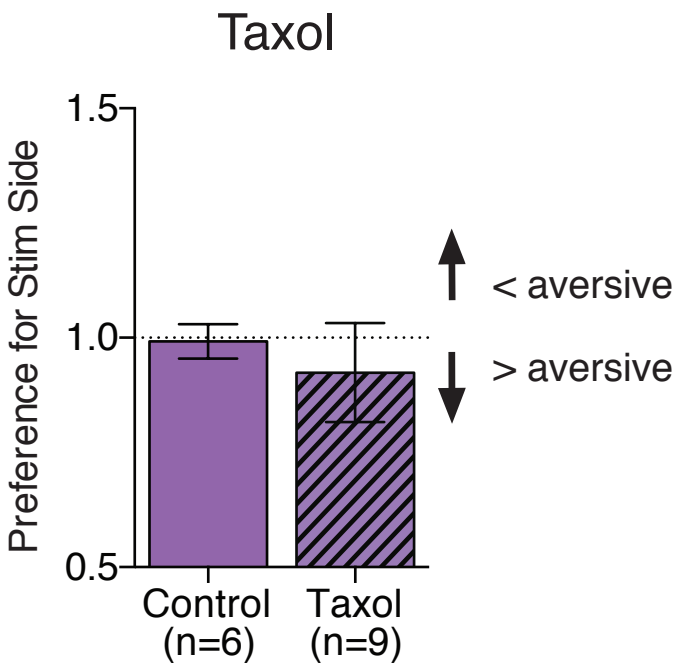
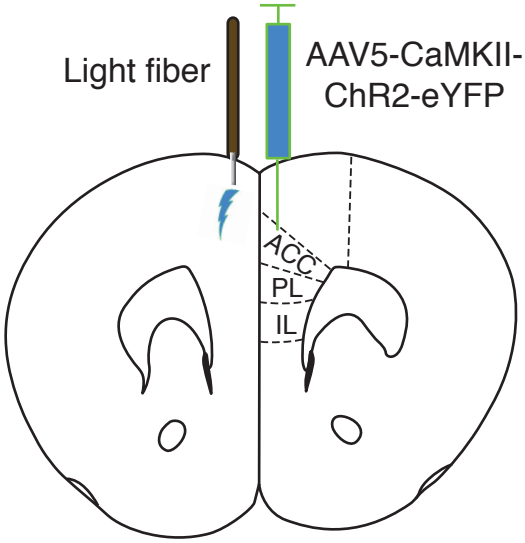
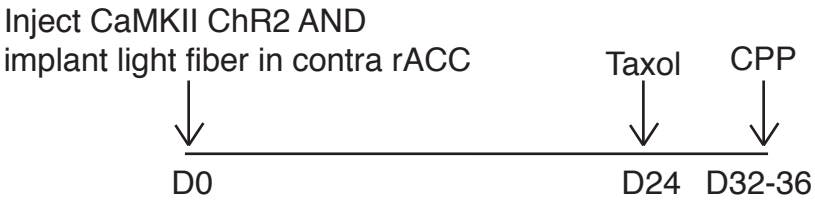

Taxol
Preference for Stim Side
1.5
1.0
0.5
< aversive
> aversive
Control
(n=6)
Taxol
(n=9)

Figure 7: Optogenetic activation of rACC terminals in contralateral rACC elicits neither preference nor aversion in controls or animals with chronic neuropathic pain.
A. Injection of AAV5-CaMKII-ChR2-eYFP in rACC and implantation of a light fiber in contralateral rACC (MD), in order to target rACC terminals in contralateral rACC. **B.** Timeline of injection, implantation and behavior. **C.** Quantification of Preference for Stim Side of control (n = 8) and Taxol (n = 5) animals. Optogenetic activation of rACC terminals in contralateral rACC elicits neither aversive nor preference in either control or animals with chronic neuropathic pain (*, $p < 0.05$ by unpaired t-test.) Errors represent S.E.M.

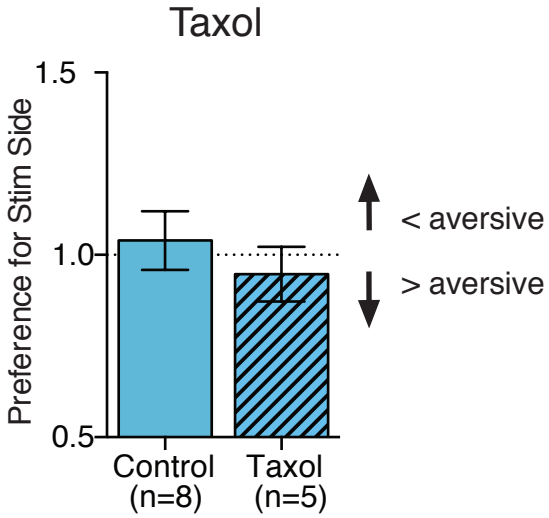
A.


Light fiber
AAV5-CaMKII-
ChR2-eYFP
ACC
PL
IL

B.


Inject CaMKII ChR2 AND
implant light fiber in contra rACC
Taxol
CPP
D0
D24
D32-36

C.


Taxol
Preference for Stim Side
1.5
1.0
0.5
< aversive
> aversive
Control
(n=8)
Taxol
(n=5)

Chapter 4

Conclusions

The experiments in this thesis described neural circuits in the rostral anterior cingulate cortex (rACC) that underlie pain-related aversion. We used optogenetic tools to activate or inhibit inputs to the rACC, as well as output neurons of the rACC. Electrophysiological and behavioral techniques used in conjunction with optogenetics allowed us to closely study the interaction between these inputs and outputs of the rACC, and describe their role in pain-related aversion.

In Chapter 1, we studied and manipulated inputs from the mediodorsal thalamus (MD), a structure that has been implicated heavily in pain perception and affective processing. Behaviorally, we found that activating MD inputs to rACC elicited conditioned place aversion only in animals with chronic neuropathic pain, i.e., activating MD inputs to rACC exacerbated pain-related aversion. Physiologically, we find that excitatory responses of layer V rACC pyramidal neurons to activation of MD inputs are dramatically decreased in the setting of chronic neuropathic pain. In fact, when we calculated the ratio between excitation and inhibition currents elicited by MD inputs in layer V rACC pyramidal neurons, we found that responses of subcortically-projecting neurons (SC) shifted towards inhibition. Indeed, when we optogenetically activated MD inputs while injecting a depolarizing current pulse, spiking of the majority of SC neurons in animals with chronic neuropathic pain stayed the same or decreased. We conclude that, in the

setting of chronic neuropathic pain, MD inputs appear to form an inhibitory pathway with rACC through layer V SC neurons.

In Chapter 2, we studied and manipulated inputs from the basolateral amygdala (BLA), a structure that has been implicated heavily in aversive learning, including fear and anxiety. We found opposing, but parallel, effects of manipulating BLA inputs compared to MD inputs. Specifically, we found that activating BLA inputs elicited conditioned place preference in both animals with chronic neuropathic pain and control animals. Strikingly, we found that inhibiting BLA inputs elicited the same effects as activating MD inputs to rACC – aversion in animals with chronic neuropathic pain (i.e. exacerbated pain-related aversion). Physiologically, we found that excitatory responses of layer V rACC neurons in response to activating BLA inputs are significantly increased in the setting of chronic neuropathic pain; this is in direct contrast to the effect of activating MD inputs.

It is worth noting here that we found convergent patterns of input-specific modulation of rACC - the net inhibitory effect of MD inputs of SC neurons and the net excitatory effect of BLA inputs of SC neurons - in two different models of neuropathic pain. The spared nerve injury (SNI) model of neuropathic pain results in robust mechanical allodynia but no hypersensitivity to heat (Braz et al., 2015). In this model there is induction of the transcription factor, ATF3, in dorsal root ganglion (DRG) cell bodies of injured afferents (Braz et al., 2010) and profound microglia activation in the dorsal horn of the spinal cord (Tsuda et al.,

2003). Chemotherapy (paclitaxel)-induced neuropathy, in contrast, results in robust mechanical allodynia and thermal hyperalgesia, but is not associated with ATF3 induction or microglial activation (Braz et al., 2015). Despite these differences in peripheral dysregulation in these two models of chronic neuropathic pain, we found comparable and highly specific patterns of cellular and synaptic dysregulation at the level of the rACC when activating MD or BLA inputs. These findings suggest that there is a common cortical mechanism that drives the aversive component of pain.

In Chapter 3, we took a closer look at SC neurons of the rACC to determine whether they act as a key node in the parallel effects we described in the previous chapters - activating MD inputs and inhibiting BLA inputs both exacerbating pain-related aversion. We proposed that reduced activity of SC neurons may underlie these behavioral effects. Indeed, when we used optogenetics to directly inhibit SC neurons, we found that this manipulation elicited conditioned place aversion only in animals with chronic neuropathic pain. In an important control experiment, inhibiting another population of output neurons (IT neurons) does not elicit the same effect, indicating that SC neurons are preferentially implicated in the control of pain-related aversion.

Our findings emphasize that the rACC is a heterogeneous structure and this should be taken into account when studying its role in pain-related aversion. We suggest a model in which the affective valence of rACC representations depends

on the presynaptic source and postsynaptic targets of distinct inputs. In other words, it is the organization of rACC circuits, rather than overall level of rACC activity, that determines the affective valence of chronic neuropathic pain. It follows that novel therapeutic interventions that preferentially target specific inputs to the rACC (e.g., from the MD thalamus) or selective populations of cells within the rACC (e.g., SC neurons) will be more effective at treating pain-related aversion than non-specific interventions at the level of the rACC.

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