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Targeting the brain for pain relief

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Targeting the brain for pain relief

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Targeting the brain for pain relief

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Abstract

Neuropathic pain is a prevalent and debilitating disease that affects millions of people worldwide. To date, the first line therapy for the treatment of neuropathic pain relies on anticonvulsants, notably gabapentin. However, the neural substrates through which gabapentin exerts its effects are far from completely understood. Gabapentin, which targets the α2δ subunit of calcium channels, acts in part in the peripheral nervous system, where it decreases the excitability of primary sensory neurons, thereby reducing nociceptive signals from reaching the spinal cord. This increased excitability is presumed to underlie the central sensitization of spinal cord neurons that contributes to mechanical hypersensitivity, a hallmark of the neuropathic pain phenotype. Gabapentin can also reduce nerve injury-induced mechanical hypersensitivity by a supraspinal action, and this is presumed to result from the activation of descending inhibitory controls that regulate “pain” transmission by spinal cord circuits.

The experience of pain involves a complex mix of sensory discriminative as well as emotional features. The sensory discriminative component can be regulated by an action on ascending spinal cord transmission pathways; the emotional component involves many elements of the limbic system and particular cortical regions, including the anterior cingulate gyrus and the insular cortex. What is not known is whether supraspinal gabapentin can regulate the pain experience by modifying the emotional component independently of the initiation of descending inhibitory controls. In the following series of studies the term “pain relief” refers to the reduction of the aversiveness of the pain experience, and is distinguished from antinociception. The first series of studies in this thesis examined whether gabapentin can induce pain relief, via supraspinal circuits. We addressed this using the conditioned place preference (CPP) paradigm in which animals associate one side of an apparatus with gabapentin administration, and the other side with saline. Animals that have ongoing pain are in a

state of tonic aversiveness, and therefore show a preference for the gabapentin-paired side of the apparatus because it reduces pain-related aversiveness (pain relief), whereas uninjured animals show no preference. Using GP-CPP paradigm, we show that there is a dose-dependent action of supraspinal gabapentin that is pain relieving. Next we asked whether this pain relieving effect required initiation of descending controls to the spinal cord, and whether noradrenergic systems were involved, or if the gabapentin pain relieving effect is independent of the antinociceptive mechanism. In this second set of experiments, we examined the effect of blocking noradrenergic signaling at the level of the spinal cord on the pain relieving action of gabapentin (using the CPP paradigm). We demonstrate that supraspinal gabapentin is, in fact, no longer pain relieving when noradrenergic signaling is blocked at the spinal cord level and conclude that supraspinal gabapentin-mediated pain relief requires descending noradrenergic controls.

Our subsequent experiments focused specifically on the brain regions that regulate the aversiveness associated with the pain experience. Numerous experiments have implicated nerve injury-induced hyperexcitability of the rostral anterior cingulate gyrus (rACC) in the generation of persistent pain aversiveness. The rACC contributes to the affective, but not sensory discriminative component of chronic pain in both rodents and humans. Patients with chronic pain who have undergone lesions of the rACC no longer perceive noxious stimuli as bothersome nor experience ongoing pain, indicating that inhibition of the rACC has beneficial therapeutic effects. Unfortunately, not only are these lesions very invasive, they often also have non-selective disruption of fibers passing through this region. With a view to developing long-term regulation of rACC activity, we introduced a novel approach to enhance inhibition of the rACC in a targeted and long-lasting manner. In these studies, we transplanted embryonic precursor cells of cortical inhibitory interneurons derived from the medial ganglionic eminence (MGE) into the rACC of adult mice. The studies were performed in mice in which we induced a chemotherapy (paclitaxel)-triggered neuropathic pain. To measure ongoing pain in these animals, we used CPP in response to systemic gabapentin, which as noted above is only rewarding in animals that experience ongoing pain. We hypothesized that if the

MGE transplanted cells are indeed pain relieving, then we should expect the animals to be pain-free, and therefore not show a preference for gabapentin.

As expected, control animals that received medium, but no cells in the rACC had a large preference for the gabapentin-paired side of the apparatus, indicating that they still experienced ongoing pain. In contrast, 30 days after a receiving a transplant of MGE cells in the rACC, the paclitaxel-treated mice no longer showed a preference for the gabapentin, indicating that they no longer experienced ongoing pain. Importantly, MGE transplantation in the rACC did not reduce paclitaxel-induced mechanical hypersensitivity, indicating that there was no effect on the sensory discriminative component of the pain experience. In other words, there was no concurrent antinociceptive effect of the transplants.

In this respect, the transplants differ considerably from the supraspinal action of gabapentin, where we demonstrated a dissociation of the pain relief and spinal cord antinociceptive action. Our finding that the rACC MGE transplants successfully relieve ongoing pain is consistent with previous results concerning the contribution of the rACC to pain aversiveness, and support the idea that selective and long lasting inhibition of the rACC is a viable therapeutic strategy for the treatment of chronic pain. Unexpectedly, mice in which the MGE cells were transplanted in *both* the rostral and posterior ACC (pACC) retained a preference for the gabapentin-side of the apparatus, which suggests that inhibition of the pACC is pro-aversive and can block the behavioral effects of MGE transplantation in the rACC. This contribution of the pACC demonstrates that there must be other brain areas that influence the rACC activity and that are also engaged in the setting of neuropathic pain. Their identification will undoubtedly expand the potential targets for long-term management of neuropathic pain.

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Background

Chronic pain:

There are two major categories of chronic pain. Nociceptive pain results from tissue injury and is generally associated with inflammation. Included in these tissue injury-based chronic pains are the arthritis, most cancer pain and headache. Neuropathic pain results from nerve injury, most commonly to the peripheral nervous system. Among the many neuropathic pains are trigeminal neuralgia, postherpetic neuralgia, brachial plexus avulsion, diabetic neuropathy, chemotherapy-induced neuropathy and fibromyalgia. Chronic back pain likely has both neuropathic and nociceptive components. There are also central neuropathic pains, which can also arise after stroke, spinal cord injury or in patients with multiple sclerosis. Epidemiological studies show that the prevalence of neuropathic pain is quite variable, depending on the patient population and neuropathy type assessed. 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). Neuropathic pain has both an ongoing spontaneous component, which can be described as shooting, burning, aching or a combination and an evoked component (mechanical allodynia and thermal hyperalgesia, to both warm and cool stimuli). The pain may be localized (as occurs from hyperactivity of a single peripheral nerve) or widely distributed across multiple dermatomes.

Multidimensional nature of pain:

Pain is a multidimensional experience comprised of two main features: a sensory/discriminative component, which refers to the quality, location, and intensity of the pain; and the affective/emotional component, which refers to the aversiveness or unpleasantness of the pain. The perception of the affective component can be altered by cognitive processes, such as attention to and distraction, anticipation and the assignment of meaning to the pain (Villemure and Bushnell, 2009; Wiech et al., 2008). Human subjects that were instructed to rate the unpleasantness of a nonpainful stimulus reported higher ratings of unpleasantness when they were expected that the stimulus would be painful (Sawamoto et al., 2000). In a similar vein, clinical evidence shows that pain associated with illness is perceived as more unpleasant compared to pain that is not associated with any particular meaning (Arntz and Claassens, 2004; Van Damme et al., 2008; Whitehead and Palsson, 1998). Expectation of the magnitude of a painful stimulus can also change the perception of its unpleasantness, with higher intensities of expected pain being perceived as more unpleasant (Koyama et al., 1998). However, under acute pain circumstances, this unpleasantness is adaptive, serving as a protective signal that provides motivational power to avoid touching or moving wounded body regions or avoid stimuli and situations that are potentially harmful.

Pain unpleasantness, however, is not analogous to pain intensity. For example, in one interesting experiment, hypnotic suggestions to either increase or decrease the perceived unpleasantness of a subject's hand immersed in a hot water bath were

successful in either direction. However, hypnotic suggestions designed to influence the subject's perception of pain intensity failed to change in the suggested directions (Rainville et al., 1999). Furthermore, lesions of specific brain regions (discussed in detail below) can attenuate the perceived unpleasantness of noxious stimuli, but do not alter perception of the discriminative components of the stimulus. Taken together these studies demonstrate that the affective component of the pain experience can be dissociated from and is independent of the sensory discriminative features of the experiment.

There is also anatomical evidence for the separation of these two processing pathways. Nociceptive information that is transmitted from the spinal cord ascends to the ventroposteriolateral thalamic nucleus (VPL) and parabrachial nucleus (PB) of the brain stem. Information from VPL is then transmitted to the primary somatosensory cortex (S1), where it likely signals the sensory-discriminatve features of the pain experience. In contrast, information from the PB is transmitted to limbic structures, including the amygdala and hypothalamus, as well as to the medial and intralaminar nuclei of the thalamus. These thalamic nuclei also receive afferent projections directly from the spinal cord, but neurons in these regions are not topgraphically organized and do not code for intensity. Rather, they typically have large receptive fields and undoubtedly are more related to the affective component of the pain experience. Consistent with this conclusion, the ultimate target of these medial thalamic, hypothalamic and amygdala neurons are brain regions that process emotion, including the anterior cingulate cortex (ACC) and insular cortex (IC). The dissociation of these processing regions is

demonstrated by the fact that lesions of S1 compromise recognition of sensory discriminative aspects of the painful stimulus, whereas lesions of limbic regions reduce their unpleasantness. Below we highlight the contribution of the anterior cingulate gyrus, which is the focus of many of the studies described in subsequent chapters.

Animal Models

One of the main focuses of these experiments was to determine the mechanism(s) through which a systemic drug (gabapentin) exerts its pain relieving effect in mice with neuropathic pain and if targeted inhibition of a limbic brain region (ACC) is effective independent of an action at the spinal cord level. For these studies, we chose two different models of neuropathic pain. One is the spared nerve injury (SNI) model, which consists of a localized injury to the sciatic nerve. This injury produces a long-lasting increase in the mechanical sensitivity of the affected paw; stimuli that were once innocuous now induce a withdrawal response. This paw withdrawal behavior in response to a previously innocuous stimulus is termed mechanical allodynia. Mechanical allodynia is due to plasticity induced in the injured dorsal-root ganglia and corresponding spinal cord circuits, which results in a sensitization of spinal reflexes. We presume that this sensitization process can occur independently of a supraspinal action, although brain mechanisms can certainly influence the magnitude of the sensitization. Here, we refer to the reversal of mechanical allodynia as “antinociception”, and is a measure of the sensory-discriminative “nociceptive” component of pain.

We also incorporated a chemotherapy-induced model of neuropathic pain. In this model, we make multiple systemic injections of paclitaxel, a common chemotherapeutic agent used in the clinic. These injections produce a long lasting-whole body mechanical allodynia (Smith et al., 2004). Again, we measure the sensory-discriminative component of pain by measuring mechanical withdrawal thresholds of the hindpaw. In addition to influencing the sensory (nociceptive) component of the pain experience, both of these preclinical models of neuropathic pain concurrently induce a state of tonic pain aversiveness. The circuits through which pain aversiveness is processed are located within limbic brain regions. The relief of this tonic aversive state is what we term “pain relief”. We cannot assess the ability of a treatment to relieve pain using paw withdrawal thresholds as a readout. Instead, we took advantage of the analgesia-induced conditioned place preference (CPP) test. In this assay, an animal associates an environment with the experience of pain relief produced by administration of an analgesic drug. If the animal experiences ongoing pain, i.e. tonic aversiveness, then it will prefer the analgesic-paired side of the chamber. If the animal is pain-free, it will show no preference. In this way, we can assess the extent to which two very different treatments, gabapentin and inhibition of the ACC (surgically or with transplants of inhibitory interneuron precursors), could induce pain relief, and if that effect was independent of the treatments antinociceptive action.

The Anterior Cingulate Cortex and pain affect

Nociceptive neurons have been identified in the ACC of humans (Hutchison et. al. 1999 (Hutchison et al., 1999), monkeys (Koyama et al., 1998), rabbits (Sikes and Vogt, 1992) and rats (Yamamura et al., 1996). ACC nociresponsive neurons are nontopographically organized, respond to multimodal noxious stimuli and have large bilateral or whole-body receptive fields (Hutchison et al., 1999; Yamamura et al., 1996). Nociceptive neurons are found in ACC layers III and V of the rat and have both pyramidal and non-pyramidal morphologies (Yamamura et al., 1996).

Human imaging studies show that acute noxious stimuli (Derbyshire, 2000; Peyron et al., 2000; Ploghaus et al., 1999) and even their anticipation (Hsieh et al., 1999; Porro et al., 2002) activate the ACC. Attending to the unpleasantness of a noxious stimulus (Rainville et al., 1997) also increases ACC activity, and increased activation of the ACC correlates with expectation of pain and the unpleasantness of innocuous stimuli (Sawamoto et al., 2000). The mechanisms through which the ACC, insular cortex and amygdala contribute to the tonic pain aversiveness that characterizes the chronic pain phenotype is poorly understood. Studies have shown that in humans and animals (Baliki et al., 2011; Baliki et al., 2014; Farmer et al., 2012) with chronic pain, the functional connectivity of limbic, but not sensory, brain regions are reorganized. This limbic reorganization results in the sensitization of emotion-processing brain regions, which contributes to the initiation of a tonic pain aversive state that characterizes chronic pain.

Patients who experience ongoing pain aversiveness have heightened neural activity in the anterior cingulate gyrus compared to healthy controls (Baliki et al., 2006). Consistent with this finding, lesions of the cingulum bundle in patients have been used to treat intractable pain in which there is, what Foltz referred to as a "high psychological titer" associated with their pain (Bernad and Ballantine, 1987; Foltz and White, 1962). Likewise, in preclinical animal models, the ACC becomes hyperactive following nerve injury. Potentiation of sensory responses and amplification of excitatory synaptic transmission occur in the ACC following digit amputation and peripheral nerve injury, respectively, in the rat (Wei and Zhuo, 2001; Xu et al., 2008). As in patients, inactivation of the ACC can relieve pain. Lidocaine-mediated inactivation of the cingulum bundle (Vaccarino and Melzack, 1989), excitotoxic lesions (Johansen et al., 2001; Pastoriza et al., 1996; Qu et al., 2011) and $GABA_AR$ or mu-opioid receptor agonist-mediated inactivation of the ACC (LaGraize et al., 2006; LaGraize and Fuchs, 2007) are all pain relieving in preclinical models of chronic pain. Importantly, none of these ACC manipulations affect the sensory discriminative component of pain perception. Taken together, these data suggest that inhibition of the ACC is a valuable therapeutic intervention for managing the affective component of chronic pain (which we term here, "pain aversiveness"). In Chapter 2 this question is directly addressed.

Subregions of the Cingulate Cortex:

The anterior cingulate cortex is has several anatomically identified subregions (corresponding in the human to Broadmann's Area 24a/b, 25, and 32) that are

distinguished in cytoarchitectural studies that reveal a different laminar architecture (Vogt et al., 1995a), receptor binding (Palomero-Gallagher et al., 2009), functional imaging and basal glucose metabolism (Vogt, 2009). However, not all regions that have been identified in the human have parallels in the mouse (i.e. Broadmann's Area 33). Area 24 is the agranular region of the ACC. This region receives inputs from the midline and intralaminar thalmic nuclei, including as the anteromedial (AM) (Horikawa et al., 1988) and mediodorsal (MD) thalamus, basal nuclei of the amygadala (BLA), agranular insular cortex (Mesulam and Mufson, 1982) and is where the nociceptive neurons reside in the ACC. The Area 24 also has high expression of delta (resident neurons) and mu opioid receptors (Hiller and Fan, 1996; Vogt et al., 1995b; Vogt et al., 1992) and direct injection of morphine into Area 24 is pain relieving (LaGraize et al., 2006; Navratilova et al., 2015). For the purpose of these studies, described in the subsequent chapters we have adopted the following nomenclature to describe specific regions of Area 24 subdivision of the ACC, as defined by Brent Vogt (Vogt and Paxinos, 2014). Here we refer to the rostral half ((Paxinos and Franklin, 2001) stereotaxic coordinates: bregma +1.18mm to +2.25mm) of Broadmann Area 24 as the rostral or "rACC", and the caudal half ((Paxinos and Franklin, 2001) stereotaxic coordinates: bregma +1.10mm to +0.25mm) of Area 24 as the posterior or "pACC". We did not make a dorsal-ventral distinction within this area. We are not the first to make the distinction between the rostral and caudal parts of the ACC. Functionally, excitotoxic lesions of the rACC, but not the pACC, block the aversion to intraplantar formalin but have no effect on the sensory-discriminative aspect of the formalin response. However, in the studies

described in Chapter 2, we provide the first evidence that the rACC and pACC functionally interact in a novel way concerning pain aversiveness.

Pharmacology

Neuropathic pain conditions are poorly responsive to conventional analgesics, such as opiates (Tasker, 2001) and non-steroidal anti-inflammatory drugs (NSAIDS). The difficulty in treating such pain conditions has thus led to the clinical use of antidepressants (tricyclics) for burning pain (Max et al., 1992) and anticonvulsant drugs carbamazepine (Matthews and Miller, 1972) and gabapentin (Nuerontin ®) (Backonja et al., 1998; Rowbotham et al., 1998). The predominant approach to using anticonvulsants as pharmacotherapy builds upon the similarities between neuropathic pain etiology and epilepsy. Remarkably, a post-hoc analysis of 4 clinical trials reported that 69% of patients experienced at least 50% pain relief (placebo, mean 39%) with antidepressants, and 74% of patients experienced pain relief with anticonvulsants (placebo mean 24%), indicating that these two methods of treatment look promising in some patient populations (McQuay, 2002). Given the complexity of these chronic pain conditions, it is not surprising many preclinical models have been developed. In the studies described in this thesis, I have used two very different models that mimic the neuropathic pain provided by traumatic nerve injury and another by chemotherapy.

Gabapentin:

Despite the anticonvulsant gabapentin showing clinical analgesic efficacy for specific types of neuropathic pain, the neural substrates through which gabapentin works to induce pain relief remain largely undefined. Gabapentin and its closely related analogue pregabalin were originally designed as GABA mimetics, and so they were thought work by binding GABA receptors/transporters in the peripheral and central nervous system. However, multiple studies have shown that this is not the case. Instead gabapentinoids bind to the a2δ-1 subunit of voltage-gated calcium channels (Gee et al., 1996). In mice with a single-point mutation in the a2δ -1 subunit, gabapentinoids are no longer effective, either as an anticonvulsant or at reversing nerve-injury induced mechanical allodynia (Field et al., 2006). Conversely, transgenic overexpression of a2δ-1 is associated with an increased incidence of pain behaviors and hypersensitivity to mechanical and thermal stimuli in uninjured mice. a2δ-1 overexpressor mice also displayed increased responsiveness to the antinociceptive effects of gabapentin (Li et al., 2006). It is also noteworthy that gabapentin is ineffective at blocking acute nociception. Gabapentin has no effect on hot plate withdrawal thresholds (Laughlin et al., 2002), tail-flick withdrawal latency (Hunter et al., 1997; Shimoyama et al., 1997), paw pressure test, or radiant heat withdrawal thresholds (Field et al., 1997; Urban et al., 2005; Yoon and Yaksh, 1999). However, gabapentin has antinociceptive action, when given systemically, intrathecally or orally, in many preclinical models of neuropathic pain, including spinal nerve ligation (Hunter et. al. 1997; Urban et. al. 2005; Hwang et. al. 1997), partial sciatic nerve ligation (Patel et al., 2001), chronic constriction injury

(Coderre et al., 2005; Hunter et al., 1997), diabetic neuropathy (Field et al., 1999), postherpetic neuralgia (Chen and Pan, 2005), and trigeminal neuralgia (Christensen et al., 2001). Therefore, the differential effect of gabapentinoids on ongoing versus acute pain suggests that its analgesic mechanism depends on the plastic changes that the nervous system undergoes following nerve injury. Because we know that neuropathic pain models are associated with plastic changes in the peripheral nervous system and spinal cord, but also in the limbic structures of the brain, we wanted to ask if gabapentin could be pain relieving by acting upon these structures in a way that is independent of turning on antinociceptive descending controls? The experiments described in Chapter 1 will directly address this question.

Inhibitory interneuron transplantation into the ACC

In these experiments, we sought a non-pharmacological approach to providing prolonged pain relief. Because inactivation of the ACC is pain relieving in both humans and in preclinical animal models, and because the ACC excitability of the ACC is increased in the setting of neuropathic pain, we sought to increase inhibitory control in the ACC using inhibitory cell transplants that were isolated from the medial ganglionic eminence (MGE) of the embryonic mouse brain. The MGE is an embryonic structure in the developing mouse brain that gives rise to the majority of the inhibitory interneurons of the cerebral cortex (Wonders and Anderson, 2006). These progenitor cells when transplanted into a different host effectively integrate and thrive and have proven effective in preclinical mouse models of epilepsy (Alvarez-Dolado et al., 2006; Baraban

et al., 2009; Calcagnotto et al., 2010a). Our laboratory demonstrated utility of the transplanting MGE cells into adult spinal cord to treat both mechanical hypersensitivity in a mouse neuropathic pain model (Braz et al., 2012; Braz et al., 2015) and persistent scratching in neuropathic itch model (Braz et al., 2014). Electrophysiological studies demonstrated that the transplants release gamma-aminobutyric acid (GABA), receive inputs from local host neurons, and form inhibitory synapses onto local excitatory neurons of the cortex (Alvarez-Dolado et al., 2006; Baraban et al., 2009). Based on this remarkable integration, MGE cell transplants provide a promising new strategy to enhance inhibitory control in aberrant neural circuits. Chapter 2 describes the analgesic efficacy of transplanting MGE cells into the ACC, providing further evidence that hyperactivity of this region of the brain is a major contributor to the tonic pain aversiveness associated with neuropathic pain and that MGE transplants can indeed effectively ameliorate this condition.

Summary

In the following chapters we will address two primary questions:

1. Is supraspinal gabapentin pain relieving in preclinical models of neuropathic pain? And if so, does supraspinal gabapentin induce pain relief independent of antinociceptive circuits?
2. Is MGE-mediated inhibition of the ACC pain relieving in animals with neuropathic pain?

Chapter 1

The pain relieving effect of supraspinal gabapentin requires noradrenergic descending controls

Introduction

The anticonvulsant, gabapentin has significant analgesic properties in several neuropathic pain conditions, including postherpetic neuralgia, complex regional pain syndrome and diabetic neuropathy (Mellick et al., 1995; Rosenberg et al., 1997; Rosner et al., 1996; Rowbotham et al., 1998). Gabapentin alleviates mechanical allodynia, thermal hyperalgesia, and spontaneous pain in patients and is also antinociceptive in several preclinical models of neuropathic pain. Gabapentin reduces Ca^{2+} currents by hindering trafficking of α2δ to the cell membrane, thereby reducing the number of functional VGCCs available (Hendrich et al., 2008). The presence of less calcium channels available at the cell membrane greatly reduces the likelihood of calcium-mediated neurotransmitter release from the affected terminals, essentially silencing the neurons that are targeted by gabapentin.

Gabapentin is not effective in treating all types of nerve-injury-induced pain, and there are preclinical studies suggesting that these differential effects reflect different influences of the particular nerve injury on the α2δ-1 subunit. For example, α2δ-1 subunits are upregulated in the dorsal root ganglion (DRG) and spinal cord of rats with mechanical hypersensitivity following a variety of traumatic peripheral nerve injuries,

mice treated with paclitaxel, and in a preclinical model of diabetic neuropathy (Gauchan et al., 2009; Luo et al., 2002). Importantly, the anti-allodynic effects of intrathecal gabapentin were only seen in animals in which there was α2δ-1 upregulation. Notably a behavioral phenotype was not seen in the vincristine model of neurotoxicity, an injury not associated with α2δ-1 upregulation (Gauchan et al., 2009; Luo et al., 2002). Luo and colleagues (2001) also reported that the α2δ-1 subunit is not induced in the spinal cord and DRG after dorsal rhizotomy, indicating that damage to the peripheral, but not to the central branch, mediates this molecular change (Luo et al., 2001). The conclusion from these studies is that gabapentin is most effective in neuropathic pain states in which there is upregulation of the α2δ subunit in pain-related circuits.

Nerve injury also likely induces expression of the α2δ-1 subunit at supraspinal sites. For example, supraspinal gabapentin is only antinociceptive in nerve-injured animals. In an acute preparation of locus coeruleus (LC) slices taken from nerve-injured animals, there is gabapentin-mediated blockade of local GABAergic inhibitory interneurons, which presumably leads to a disinhibition of noradrenergic neurons that project to the spinal cord dorsal horn (Takasu et al., 2008). Of particular interest, the gabapentin-mediated inhibition of GABAergic neurons in the LC was not seen in uninjured animals, indicating that in the setting of nerve injury an upregulation of α2δ-1 in the LC may be required for gabapentin's analgesic effect at this site. These findings are consistent with other reports demonstrating that supraspinal administration of gabapentin engages a descending bulbospinal noradrenergic inhibitory pathway, but only in nerve-injured

animals. The result of the disinhibition (and release of the descending inhibitory control) is a significant reversal of mechanical allodynia associated with nerve injury.

Pharmacological studies also demonstrated a noradrenergic basis for the gabapentin-induced descending control. For example, inhibition of spinal α2-adrenoreceptors counteracts the anti-allodynic effect of supraspinal gabapentin (Hayashida et al., 2007; Takasu et al., 2006; Tanabe et al., 2008). Furthermore, depletion of monoamine production from descending projections by spinal administration of the noradrenergic neurotoxin, 6-hydroxydopamine (6-OHDA), prior to a peripheral nerve injury, greatly reduced the anti-allodynic and anti-hyperalgesic effects of supraspinal gabapentin (Takeuchi et al., 2007; Tanabe et al., 2005). Finally, neurochemical evidence from injured rodent models and patients showed that supraspinal and systemic gabapentin administration significantly increases spinal cord tissue and CSF levels of noradrenaline and its metabolites, respectively (Hayashida et al., 2007; Takeuchi et al., 2007).

The studies discussed thus far have described gabapentin's antinociceptive ability – that is, its ability to reduce the hyperexcitability of spinal cord circuits produced by nerve injury (anti-allodynia). What is not established is the extent to which the relief of pain by gabapentin also involves reduction of the excitability of supraspinal circuits that are more relevant to generation of the pain percept. In other words, what remains to be determined is whether gabapentin's analgesic action has a dual basis, blockade of the transmission of the injury message by an action in the spinal cord, and/or reduction of the supraspinal circuits that ultimately generate the pain percept, which includes the

affective, emotional component of the experience. In the following section, we refer to "pain relieving" when there is a documented supraspinal mechanism that operates to attenuate the conscious aversiveness of the pain percept.

To determine if gabapentin is pain relieving we tested injured and uninjured mice using a conditioned place preference (GP-CPP) paradigm. In a test with gabapentin, animals are placed into a two-chambered apparatus in which they learn to associate one chamber with gabapentin, and the other with vehicle. If the injured but not the uninjured, animals show a preference for the gabapentin-paired chamber, then we conclude that the animals found gabapentin administration to be a positive experience (i.e. pain relieving). Using this approach, we can assess the pain relieving effects of supraspinal gabapentin. We can also determine if any observed pain relief depends on the noradrenergic pathway to the spinal cord, or if there are also independent supraspinal sites of action and possibly different pharmacological bases of the pain relief.

Results

Systemic gabapentin is pain relieving in paclitaxel-treated mice

First, we asked if systemic gabapentin can relieve pain in a chemotherapy (Taxol) model of neuropathic pain. As the CPP test can be complicated if the pharmacological agent tested has rewarding properties, we first determined whether gabapentin is inherently rewarding. This conclusion would be drawn if uninjured animals show a preference for the drug when tested in the CPP paradigm. If only nerve-injured animals

show a preference for the drug-paired chamber during CPP, then we can conclude that the change in preference occurred only because of the drug's pain relieving properties.

Paclitaxel (1.0 mg/kg, i.p.) or vehicle (40% DMSO, i.p.) was administered every other day for 4 days. One week after the last injection, all animals were assayed for gabapentin-induced CPP (GP-CPP). The mice received systemic gabapentin (i.p. 30mg/kg) 45 min prior to being restricted to one chamber of the CPP apparatus, and received systemic saline when restricted to the other chamber. **Figure 1** illustrates that paclitaxel-injected animals showed a positive change in preference (CPP score = 191.4 ± 71.73, Student's *t*-test, p = 0.0294, N = 6) for the gabapentin-paired chamber. The uninjured animals showed no change in preference for the gabapentin-paired chamber (CPP Score = 16.47 ± 21.60, Student's *t*-test, p = 0.6398 N = 5). We conclude that systemic gabapentin (30mg/kg) is pain relieving, and because mice without nerve injury showed no change in their preference for the gabapentin-paired chamber, we conclude that gabapentin is not inherently rewarding.

Next, we performed experiments to determine the site of action of gabapentin-mediated pain relief, focusing on supraspinal circuits. In these experiments we implanted animals with a cannula that targeted the lateral ventricle. We studied two groups, which received either paclitaxel (N = 8) or vehicle (N = 10). To assess accurate targeting of the ventricle, six days after cannula implant, the animals underwent an angiotensin II-induced drinking test (ATII test). Three days later, the mice in which ATII relatively induced drinking, were tested in the CPP paradigm. Gabapentin was administered

directly into the lateral ventricle (**Figure 2**; 100µg/1µL, i.c.v.). Immediately after receiving this injection, the mice were placed into one of the chambers. Again, i.c.v. gabapentin induced a significant change in preference for the GP-paired side of the box, but only in paclitaxel-treated mice (CPP Score = 113.5sec ± 59.49; N = 8, Student's *t*-test, p = 0.0156). Based on this experiment, we conclude that gabapentin indeed has a pain relieving action when administered supraspinally.

Supraspinal gabapentin administered at a dose that does not reverse mechanical allodynia does not relieve pain in paclitaxel-treated mice.

As noted above supraspinal gabapentin in rodents can reverse peripheral nerve injury-induced mechanical allodynia by engaging inhibitory noradrenergic projections to the spinal cord (Takasu et al., 2006; Takeuchi et al., 2007; Tanabe et al., 2008). Here we determined whether there is a gabapentin-mediated supraspinal pain mechanism that is independent of these descending controls. In these experiments we asked whether there is an i.c.v. dose of gabapentin that does not initiate descending controls (i.e. increase mechanical thresholds) but is still pain relieving (i.e. induces a change in place preference).

We first established a dose-response for the effect of i.c.v. gabapentin on mechanical thresholds in nerve-injured mice (**Figure 3**). Mechanical thresholds were assessed one week after the last paclitaxel injection. Immediately after, the mice received i.c.v. gabapentin at 30µg (N = 14), 50µg (N = 8), or 100 µg /µL (N = 9) or vehicle (saline, N =

8). Figure 3 illustrates that i.c.v. gabapentin (50 µg and 100 µg) significantly reversed the paclitaxel-induced mechanical allodynia. As the 30 µg did not (Figure 3; One-way ANOVA, Tukey's multiple comparison's test, BL vs. 30µg GP, $p < 0.01$), we conclude that this dose does not initiate sufficient anti-nociceptive descending controls. Having established this dose response curve, one week later, we repeated the 30µg i.c.v. dose in the GP-CPP paradigm. In this case, we paired one chamber of the CPP apparatus with 30µg i.c.v. gabapentin. **Figure 4** illustrates that these paclitaxel-treated animals (N=8) did not show a preference for the gabapentin-paired side (**Figure 4**; CPP Score = -27.00 ± 81.49, Student's *t*-test, $p = 0.6281$). Therefore, we conclude that 30µg i.c.v. gabapentin is below the threshold for relieving pain as measured by CPP. In other words, in order for i.c.v. gabapentin to be pain relieving it must be given at a dose that also initiates descending controls that have an anti-nociceptive effect by an action at the level of the spinal cord.

The pain relieving effect of supraspinal gabapentin requires noradrenergic descending controls

In the next set of experiments we examined the mechanisms through which supraspinal gabapentin exerts its antinociceptive effects. Because the paclitaxel model of neuropathic pain induces hypersensitivity over fore and hind limbs it would be difficult to reduce completely an antinociceptive effect by intrathecal drug administration, which typically only targets the lumbosacral spinal cord. For this reason, in these experiments we used the sciatic nerve injury (SNI) model of neuropathic pain, in which the animal develops a significant mechanical hypersensitivity that is restricted to the injured

hindpaw. In this setting, after administering i.c.v. gabapentin at an antinociceptive dose (based on the paclitaxel study), we blocked noradrenergic signaling in the corresponding level of the spinal cord and determined whether there is a demonstrable pain-relieving effect even when the antinociceptive (spinal) effect of gabapentin is blocked. In other words, this experiment tests whether engaging descending controls is necessary to induce pain relief in the CPP test or whether supraspinal gabapentin engages a separate analgesic neural circuit in the brain, one that is independent of the circuit that engages descending inhibitory controls.

To block descending noradrenergic signaling, we administered the α2-adrenergic receptor antagonist, yohimbine (5.0μg/5μL) intrathecally. The yohimbine was administered at the same time that we injected an antinociceptive dose of i.c.v. gabapentin (50μg). As noted above, this dose of gabapentin reliably engages descending controls and is antinociceptive in a test of mechanical threshold. We first compared the effect of intrathecal (i.t.) yohimbine (N = 8) or vehicle (saline, N = 8) on the anti-allodynic effect of 50μg i.c.v. gabapentin in animals that underwent spared nerve injury (SNI). Control animals received i.c.v. gabapentin plus intrathecal saline (**Figure 5**). **Figure 5a** shows that gabapentin returned mechanical thresholds of the injured paw to pre-injury, baseline levels (2-way RM ANOVA, Sidak's multiple comparison test, $p < 0.0001$, when compared to post-SNI threshold). In contrast, in animals that received i.t. yohimbine, we only recorded a partial reversal of mechanical thresholds (2-way RM ANOVA, Sidak's multiple comparison test, n.s. when compared to the post-SNI threshold). Importantly, neither i.c.v. gabapentin nor i.t. yohimbine

administered alone altered the mechanical threshold of the uninjured paw (**Figure 5b;** 2-way RM ANOVA, Sidak's multiple comparison test, n.s.), which demonstrates that there is not a tonic descending inhibitory control that can be enhanced by gabapentin.

Next, we repeated the gabapentin/yohimbine experiments, but here we used the CPP paradigm to monitor the pain relieving effects of suprapinal gabapentin. These experiments tested the hypothesis that although gabapentin clearly engages a noradrenergic descending control system (anti-nociception), there may exist supraspinal pain relieving circuits that are independent of the inhibitory controls exerted at the level of the spinal cord. Here, when animals were restricted to one chamber and given i.c.v. gabapentin, they also received either i.t. yohimbine (5ug/5uL, N = 6) or i.t. vehicle (saline, N = 8). When mice received i.c.v. saline in the other chamber, they also received i.t. saline. Figure 6 shows that mice that received i.t. saline paired with i.c.v. gababentin had a large preference for the GP-paired chamber (**Figure 6**; CPP Score = 92.10 ± 24.76, Student's *t*-test, p = 0.0360), a finding that is consistent with our earlier observations showing that gabapentin is pain relieving. In contrast, animals that received i.t. yohimbine paired with i.c.v. gabapentin lost the preference for the GP-paired chamber (CPP Score = -16.17 ± 12.41, Student's *t*-test,p = 0.3927). Collectively, these data indicate that the pain relieving effect of i.c.v. gabapentin is dependent upon the engagement of a descending, noradrenergic inhibitory control system, one that operates at the level of the spinal cord. Based on these experiments, we conclude that gabapentin does not exert a pain relieving action at the level of the brain without concurrently engaging descending inhibitory controls.

Discussion

The anticonvulsant drug gabapentin and its closely related analogue, pregabalin, are effective in the management of different forms of neuropathic pain. The analgesic efficacy of these drugs appears to be dependent on an upregulation of the molecular target of gabapentin, namely a2δ-1, in particular areas of the nervous system following injury. In this series of studies, we first determined the extent to which supraspinal mechanisms are responsible for the analgesic efficacy of gabapentin, and if these mechanisms are dependent on disinhibition of descending inhibitory controls. Preclinical studies have clearly established that supraspinal gabapentin is antinociceptive (i.e. in the setting of injury, gabapentin inhibits withdrawal reflexes in response to an innocuous stimulus). However, clinical reports document that gabapentin, in fact, relieves pain, i.e. exerts an effect beyond antinociception. We believe that our findings are the first to demonstrate behaviorally that gabapentin is also pain relieving in animals.

We also provided evidence that gabapentin's pain-relieving properties require initiation of descending noradrenergic controls, which alter processing of nociceptive messages at the level of the spinal cord. Finally, we asked whether gabapentin-induced pain relief requires a concurrent antinociceptive activity or whether there is an independent supraspinal pain-relieving circuit. From the present results, we conclude gabapentin's pain relieving properties requires initiation of noradrenergic inhibitory controls exerted at the level of the spinal cord. Based on other studies, we suggest that the locus

coeruleus is the origin of these supraspinal gabapentin-mediated antinociceptive controls.

In our studies we use the term “nociception” when we refer to spinal cord circuits that process inputs from the periphery, as well as the spinal cord sensitization processes that manifest as mechanical allodynia. The latter is measured behaviorally in animals as a reflex response, in which the animals withdraw the hindpaw in response to a normally innocuous mechanical stimulus. We found that systemic gabapentin indeed has antinociceptive properties, in that it reverses the allodynic phenotype produced by partial nerve injury. However, because these behavioral readouts are based on spinal reflexes, they are not indicative of a pain percept having occurred. It follows that one cannot conclude that the animal is experiencing pain. Clearly, other behavioral endpoints are required in order to draw conclusion about the pain relieving properties of a particular drug.

In the present study we used the conditioned place preference (CPP) paradigm in which an animal associates one chamber of an apparatus with a drug that has no inherently rewarding properties (in our studies, gabapentin) (Andrews et al., 2001). A change in the animal’s preference for the drug-paired side indicates that the animal found the drug pain relieving i.e. it is an analgesic. The rewarding property of pain relief has been shown to require activity in the ventral tegmental area (VTA), the origin of mesolimbic dopamine (DA) neurons. The VTA sends dopaminergic projections to the nucleus accumbens (NAc), which release DA in response to rewarding stimuli. Peripheral nerve

block in animals with incisional pain, evokes DA release in the NAc and this release correlates with preference for the chamber that was paired with the peripheral nerve block. Inhibition of VTA activity or NAc DA receptors blocks CPP in response to the nerve block. Taken together these findings indicate that analgesic treatments are "rewarding", but only in injured animals (Navratilova et al., 2012). This finding is consistent the report showing showing that gabapentin does not induce dopamine release in the NAc of uninjured animals (Andrews et al., 2001), whereas oral-gabapentin increases NAc DA release in animals with peripheral nerve injury (Xie et al., 2014). Therefore, the rewarding aspect of pain relief, as measured by CPP, is a reliable assay for evaluating the effect of a treatment again persistent aversiveness caused by nerve injury.

The main finding of the present study is that the pain relieving effect of supraspinal gabapentin requires noradrenaline release, and therefore an antinociceptive action at the level of the spinal cord. Interestingly, this conclusion differs considerably from the proposed supraspinal mechanism of morphine-induced analgesia. Navratilova and colleagues reported that that low-dose (0.5mg/kg) i.v. morphine does not reverse mechanical allodynia in nerve-injured rats. However, this dose of systemic morphine successfully induced CPP in injured, but not uninjured, animals (Navratilova et al., 2015). Furthermore, pharmacological blockade of μ-opioid receptors in the anterior cingulate cortex (ACC) blocked morphine-induced CPP, but not at a higher dose (4mg/kg), a dose that is rewarding in both injured and uninjured rats. These data demonstrate that morphine can engage ACC circuits to induce pain relief, and that this

effect is independent of turning on descending inhibitory controls that act at the spinal cord (antinociception).

The present study is not the first to demonstrate that gabapentinoids act supraspinally. Supraspinal gabapentin attenuates the release of GABA in LC slices (Takasu et al., 2008). As GABAergic neurons in the LC regulate the activity of LC noradrenergic projection neurons that target the spinal cord, it follows that GP-mediated inhibition of these interneurons will result in disinhibition of noradrenaline (NE) release. The result is an increase of NE-mediated antinocieption at the level of the spinal cord. Interestingly, gabapentin was only able to suppress $GABA_A$-mediated IPSCs in LC slices in animals that had undergone partial nerve injury. Because the α2δ -1 subunit is highly expressed in the LC (Cole et al., 2005), it has been postulated that just as in peripheral components of the nervous system (DRG), α2δ -1 might be up-regulated on LC GABAergic neurons following nerve injury thereby contributing to an injury-specific action of gabapentin in reducing LC GABA release (Tanabe et al., 2008).

As noted above, there is evidence that supraspinal gabapentin initiates descending controls to the spinal cord, but whether engaging these descending controls is necessary for the ability of gabapentin to induce pain relief has not been studied. Here we show that the pain relieving action of supraspinal gabapentin clearly requires an antinociceptive action at the level of the spinal cord. Thus, gabapentin was no longer pain relieving in the presence of the spinal α2-receptor antagonist, yohimbine. This finding agrees with earlier reports that the antiallodynic effects of systemic and

supraspinal gabapentin were strongly suppressed following intracisternal 6-OHDA, which depletes monoaminergic terminals in the brainstem and spinal cord. Intrathecal administration of yohimbine also significantly reduced the anti-nociceptive effects of systemic gabapentin (Tanabe et al., 2005). On the other hand, intrathecal administration of the α1-adrenergic receptor antagonist had no effect. Subsequent studies (Takasu et al., 2006), including the present one, confirmed that blockade of spinal α2-receptors greatly attenuates the antinociceptive effects of supraspinal gabapentin. Here we demonstrate not only that pharmacological blockade of spinal α2-receptors attenuates the antinociceptive effects of supraspinal gabapentin, but also that the same treatment prevents gabapentin's pain relieving property (as measure by GP-CPP). That is, in the absence of descending noradrenergic control, supraspinal gabapentin is no longer antinociceptive and its pain relieving action are abrogated. Therefore, consistent with other studies, our behavioral and pharmacological analyses demonstrated that i.c.v. gabapentin evokes norepinephrine and that this release is likely a major contributor to the antinociceptive action of supraspinal gabapentin. Though our findings are in agreement with others (Takeuchi et al., 2007), these studies did not assess if this norepinephrine release into the spinal cord was indeed pain relieving for the animal, nor did they show if it was necessary for the supraspinal analgesic action of gabapentin. Based on our present findings, we conclude that norepinephrine release into the spinal cord and its likely inhibition of the transmission of nociceptive messages to the brain is required for the pain relieving effect of supraspinally-administered gabapentin.

Chapter 1

Materials and Methods

Mouse Lines:

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California San Francisco. All animals used were C57BL6 male mice between 6 and 8 weeks of age (22g-30g).

Neuropathic pain models

Sciatic nerve injury: To produce mechanical hypersensitivity we used the spared nerve injury (SNI) neuropathic pain model as described previously (Shields et al., 2003). Briefly, mice were anesthetized with 2% isofluorane and then we made a small incision on the left thigh, which exposed the sciatic nerve proximal to its trifurcation. Using 8-0 silk suture (Ethicon, Summerville, NJ), a tight ligature was tied around the common peroneal and sural nerve branches of the sciatic nerve, followed by their transection and removal of a 1mm segment distal to the ligature. This procedure spares the tibial nerve. Overlying muscle and skin layers were close separately with 6-0 silk suture and staples (Harvard Apparatus, Holliston, MA), respectively.

Paclitaxel model of neuropathic pain: To produce mechanical and heat hypersensitivity that mimics a chemotherapy-induced neuropathic pain condition, we used the paclitaxel model (Smith et al., 2004). Adult wild-type mice were injected with 1.0 mg/kg of paclitaxel (Sigma, St. Louis, MO), dissolved in 40% Dimethyl Sulfoxide (DMSO) saline. These paclitaxel injections were repeated 4 times, every other day.

Surgical procedures

i.c.v. cannulation: Mice were deeply anesthetized with 2% isofluorane and placed securely in a stereotaxic instrument (Model 1900 Kopf). Scalp hair was removed with Nair, the scalp cleaned with betadine and ethanol, after which a midline skin incision was made. A burr hole was made for stereotaxic targeting of the left lateral ventricle: -0.875mm, -0.3mm, -2.7mm. Next we implanted a cannula (26GA, Plastics One Inc., S.W. Roanoke, VA) that was secured to the skull with super glue and dental cement. The cannula was capped when access was not required. Mice were housed together following surgery, but food carriers were removed to avoid damaging the implant. In this setting food was provided on the floor of the cage.

Angiotensin II Test: To confirm proper placement of the cannula, we injected angiotensin II (ATII, 0.1µg/µL in saline) using tubing connected to a 10µl Hamilton syringe (33GA, Plastics One Inc., S.W. Roanoke, VA). The injection system was first filled with mineral oil and then 5.0 µL of ATII was front-loaded into the injection piece, which was then inserted into the cannula. After manually injecting 1.0 µL ATII, the mouse was placed into an empty cage that contained only a petri dish of water. One minute later, we measured the length of each drinking bout, over the next 5 minutes. Animals that drank for at least 9 seconds were considered to have an on-target cannula implant.

Behavior

Conditioned Place Paradigm: For the conditioned place preference paradigm (CPP), we used a 3-chambered apparatus custom designed apparatus (Tap Plastics). Each chamber had different visual (dots vs. stripes), olfactory (lemon vs. vanilla extract) and flooring (smooth vs. rough), which that distinguished the two end chambers. The middle chamber, which served as the neutral chamber, had d no visual, tactile or odor cues that distinguished it. During habituation sessions, and on Pretest or Test days, the mice were always placed first into the neutral chamber.

On **Day 1**, the mice were habituated with full access to all parts of the test apparatus in the afternoon (3:30PM – 4:00PM). On **Day 2**, the mice were habituated to the environment in the morning (9:00AM – 9:30AM), and in the afternoon their baseline preference for each chamber was recorded, for 30 min ("Pretest") Importantly, in establishing whether there was a preference for each chamber, only the second 15 min of the video was scored. After Pretest readings were determined, a presumptive analgesic "pain reliever" (e.g., gabapentin) was paired with the less preferred chamber, and saline was paired with the more preferred chamber. In the conditioned place aversion (CPA) paradigm, an aversive stimulus (viz., intraplantar formalin) was paired with the more preferred chamber. On the conditioning days, **Day 3 and 4**, morning (9:00AM), the control substance (i.e. saline) was administered – the mice were injected i.p. with saline (volume equivalent to that required for the drug injection) and 45 min later were restricted for 30min to one of the two chambers. In the afternoon (3:30PM)

we performed conditioning sessions for the experimental drug (i.e. gabapentin) In this session the mice were injected with the test drug and then restricted to the other chamber for 30 min. We injected gabapentin either intraperitoneally 45 min before the mice were placed into the chamber or icv. immediately before the animal was placed into the chamber. On **Day 5**, the "Test" day, the mice were placed into the middle chamber and allowed to roam freely between the 3 chambers of the apparatus after which their preference for each chamber during the second 15 min of the trial was recorded.

To calculate the CPP Score, we subtracted the time (seconds) spent in each chamber of the box on the Pretest day from that of the Test day (**CPP Score** = Test-Pretest). CPP Scores for each chamber (i.e. drug-paired side or vehicle-paired side) for animals within each experimental group (i.e. lesioned vs. no lesion) were pooled. Within each group (sham surgery vs. experimental surgery) CPP scores for the drug paired chamber vs. vehicle-paired chamber were analyzed with paired t-tests.

Mechanical threshold testing: For all groups of animals tested, we recorded 3 days of baseline mechanical sensitivity before any surgical procedures were performed. Animals were habituated on a wire mesh for 2 hours, after which used von Frey filaments (1.65, 2.44, 2.84, 3.22, 3.61, 3.84, 4.08, 4.31g) to measure mechanical withdrawal thresholds, using the up-down method. For each animal, we compared mechanical thresholds taken one week after nerve injury to the average of 3 pre-surgery baseline readings. If a particular animal's mechanical threshold was reduced by at least

50% relative to its average baseline, then it was considered to have nerve-injury induced mechanical allodynia.

Pharmacological experiments

i.c.v. Gabapentin: After we determined mechanical thresholds in the animals that underwent either the SNI or paclitaxel procedures, the cannula dummy cap was removed and gabapentin (or saline vehicle), was delivered i.c.v. (30µg, 50µg or 100µg/µL) using a 10µL using a Hamilton syringe attached to tubing with a Plastics One injector piece that fit the length of the cannula (2.7mm deep). After 10s the cap was replaced to ensure no fluid backflow. The investigator was blind to i.c.v drug administered (saline vs. gabapentin).

For experiments testing the effect of spinally administered noradrenaline receptor antagonist on the ability of supraspinal gabapentin to reverse mechanical allodynia, we injected yohimbine (5µg/5µL) intrathecally into the lumbar space with a 30 gauge needle attached to a 10µL Hamilton syringe. The yohimbine was injected immediately prior to the i.c.v. gabapentin. The experimenter was blind to the intrathecal drug injected (saline vs. yohimbine).

Supraspinal GP-CPP: As described in the **Conditioned Place Paradigm** section, animals were habituated for two days to the test environment, after which their baseline preference for each chamber of the CPP apparatus was recorded. For Pretest and Test

days, the animal's preference was documented only during the last 15 min of the 30 min spent in the apparatus. For the conditioning days of this experiment, the control group received i.c.v. gabapentin/i.t. saline paired with one chamber, and i.c.v. saline/i.t. saline with the other chamber. The experimental group received i.c.v. gabapentin/i.t. yohimbine paired with one chamber, and i.c.v. saline/i.t. saline paired with the other chamber.

One week after nerve injury (SNI or paclitexel) and during conditioning days 1 and 2, for the morning conditioning sessions, the mice were lightly anesthetized (1% isofluorane) and then received an i.t. injection of saline (5.0 μL). Once awake, the animals received an i.c.v. injection of saline (1.0 μL), after which they were immediately restricted to one of the conditioning chambers, for 30 min. In the afternoon conditioning session, the mice were again lightly anesthetized (1% isofluorane) after which they received i.t. yohimbine (5μg/5μL) or vehicle (saline). Once awake, we injected gabapentin (50μg/μL) i.c.v. and then immediately restricted the mice to the opposite conditioning chamber for 30min. On the test day, we did not administer drugs. Rather, the mice were placed into the CPP box and allowed to roam freely. To calculate the CPP Score, we subtracted the time spent in each chamber on the Pretest day from the time spent in the corresponding chamber on the Test day (**CPP Score** = Test – Pretest).

Figure 1: Systemic gabapentin is pain relieving in paclitaxel-treated mice. Histogram shows the CPP Score for uninjured (black bars, n = 5) and paclitaxel-treated mice (red bars, n = 6) for the saline-paired (open bars) and gabapentin-paired (filled bars, 30mg/kg i.p.) sides. Only paclitaxel-treated mice show a preference for the gabapentin-paired side of the box (*p = 0.0294; Student's *t*-test compared to the saline-paired side). Data are represented as mean ± s.e.m.

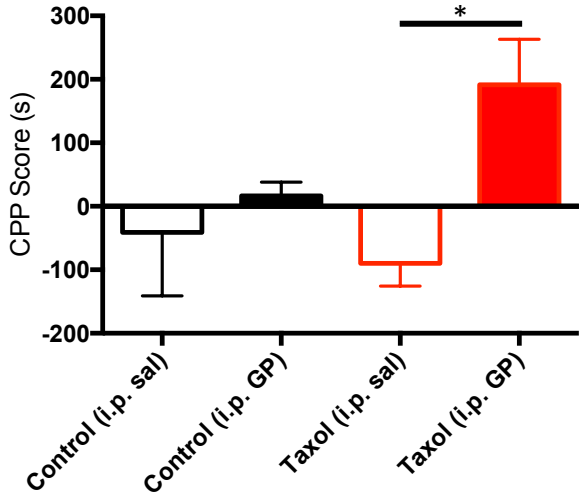

*
300
200
100
0
-100
-200
CPP Score (s)
Control (i.p. sal)
Control (i.p. GP)
Taxol (i.p. sal)
Taxol (i.p. GP)

Figure 2: Supraspinal gabapentin is pain relieving. Histogram shows the CPP Score in uninjured (black bars, n = 10) and paclitaxel-treated mice (red bars, n = 8) for the saline-paired (open bars) and gabapentin-paired (filled bars. 100µg i.c.v.) sides. Only paclitaxel-treated mice show a preference for the gabapentin-paired side (*p = 0.0156; Student's *t*-test compared to the saline-paired side). Data are represented as mean ± s.e.m.

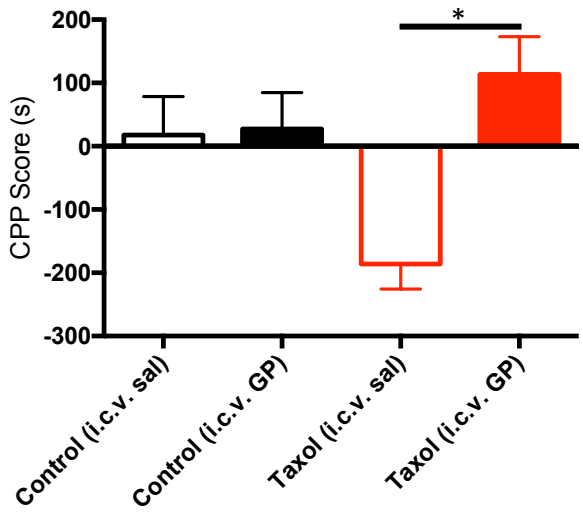

*
200
100
0
-100
-200
-300
CPP Score (s)
Control (i.c.v. sal)
Control (i.c.v. GP)
Taxol (i.c.v. sal)
Taxol (i.c.v. GP)

Figure 3: Dose-response curve for the antinociceptive effect of supraspinal gabapentin. Gabapentin was administered intracerebroventricularly at 30μg, (n = 14), 50μg (n = 8), or 100μg (n = 9) to paclitaxel-treated mice. Both the 50μg and 100μg i.c.v. gabapentin dose reverses mechanical allodynia of the hindpaw; the low-dose (30μg) does not. Saline i.c.v. had no effect on mechanical thresholds (n = 8, One-way ANOVA, Tukey's multiple comparison's test). Data are represented as mean ± s.e.m.

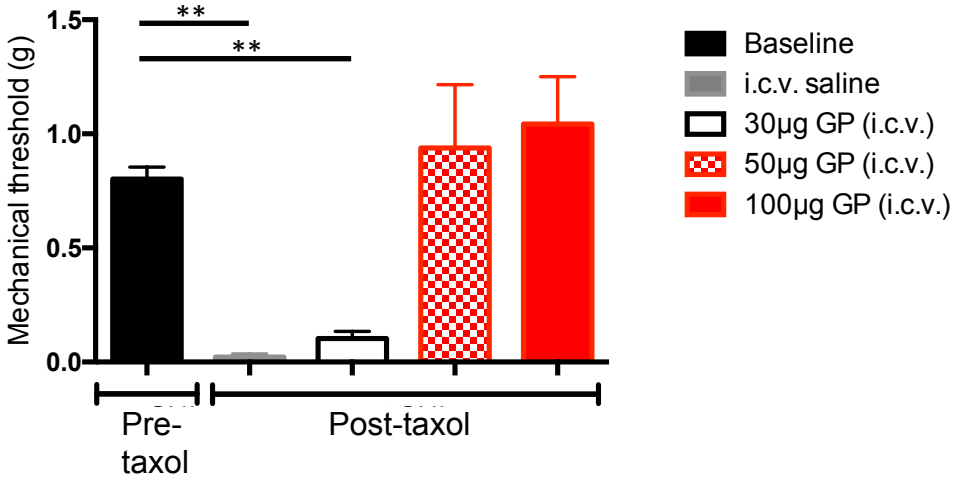

**
**
1.5
1.0
0.5
0.0
Mechanical threshold (g)
Pre-
taxol
Post-taxol
Baseline
i.c.v. saline
30µg GP (i.c.v.)
50µg GP (i.c.v.)
100µg GP (i.c.v.)

Figure 4: Low-dose supraspinal gabapentin is not pain relieving. Histogram shows the CPP Score in paclitaxel-treated mice (n = 8) for the saline-paired (open red bar) and gabapentin-paired (30μg i.c.v., filled red bar) sides. There is no significant difference in CPP score between the two sides (Student's *t*-test). Data are represented as mean ± s.e.m.

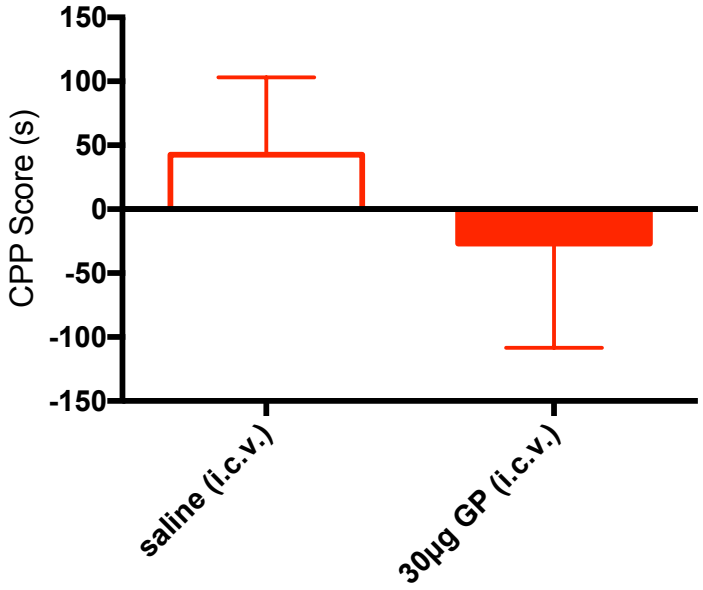
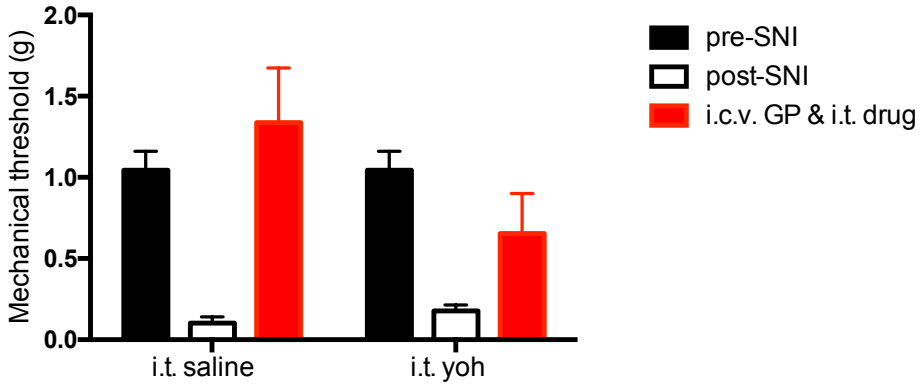

150
100
50
0
-50
-100
-150
CPP Score (s)
saline (i.c.v.)
30µg GP (i.c.v.)

Figure 5: Blocking spinal α2 receptors reduces the antinociceptive effect of supraspinal gabapentin. Histogram shows the effect of i.t. saline (n = 8) or i.t. yohimbine (5μg, n = 8) on the antinociceptive effect of supraspinal gabapentin (50μg, i.c.v.) in mice with peripheral nerve injury. **(a)** Following SNI, the mechanical threshold to induce a withdrawal response of the injured paw drops significantly (**p < 0.01) from baseline. Supraspinal gabapentin increases the withdrawal threshold, back to baseline, demonstrating an antinociceptive effect. Intrathecal saline has no effect on the antinociceptive action of supraspinal gabapentin (open black bar compared to filled red bar, ****p < 0.0001); intrathecal yohimbine partially reverses the effect of gababentin (open black bars compared to filled red bar, n.s. Two-way RM ANOVA, Sidak's multiple comparison test)**.** Mechanical thresholds of the uninjured paw are shown in **(b)**. Supraspinal gabapentin and i.t. yohimbine have no effect. Data are represented as mean ± s.e.m.

a.


Mechanical threshold (g)
2.0
1.5
1.0
0.5
0.0
i.t. saline
i.t. yoh
pre-SNI
post-SNI
i.c.v. GP & i.t. drug

b.

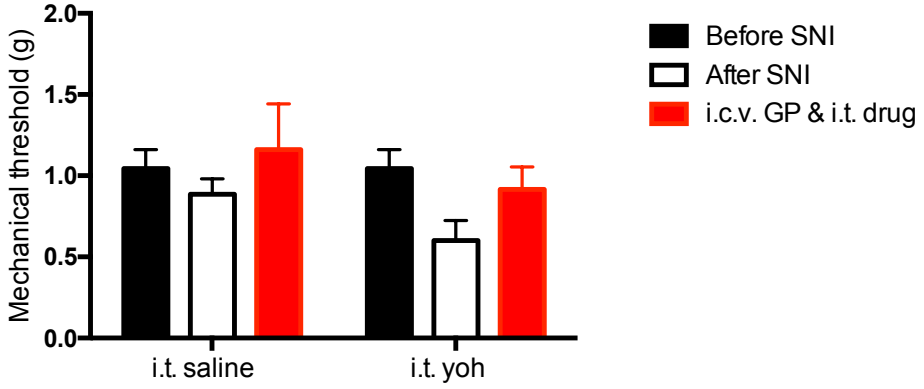
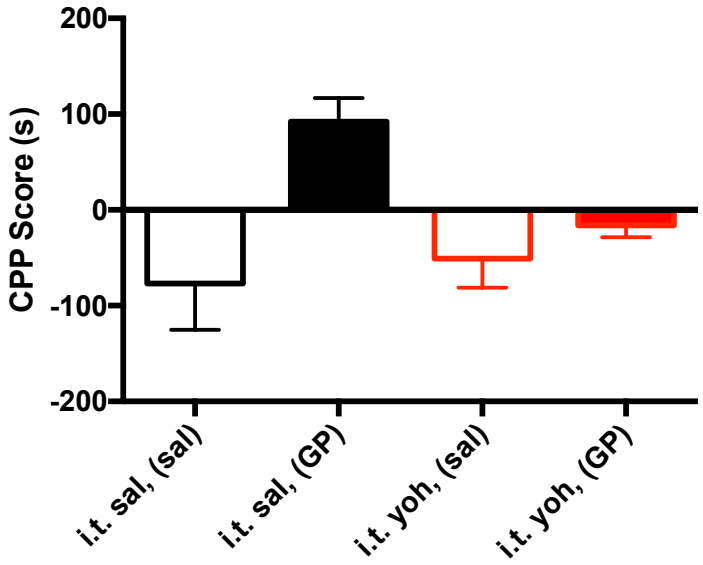

Mechanical threshold (g)
2.0
1.5
1.0
0.5
0.0
i.t. saline
i.t. yoh
Before SNI
After SNI
i.c.v. GP & i.t. drug

Figure 6: The pain relieving effect of supraspinal gabapentin requires descending noradrenergic controls. Histogram shows the CPP Score of mice that received i.t. saline (black bars, n = 8) or i.t. yohimbine (5μg, red bars, n = 6) for the saline-paired (open bars) and gabapentin-paired (50μg i.c.v., filled bars) sides. Yohimbine blocked the effect of supraspinal gabapentin. Only mice that receive i.t. saline show a preference for the gabapentin-paired side (*p = 0.0360; Student's *t*-test as compared to the saline-paired side). Data are represented as mean ± s.e.m.


200
100
0
-100
-200
CPP Score (s)
i.t. sal, (sal)
i.t. sal, (GP)
i.t. yoh, (sal)
i.t. yoh, (GP)

Chapter 2

Cell-transplant mediated inhibitory control within the rostral anterior cingulate cortex relieves the aversive properties of chronic neuropathic pain

Introduction

The development of different animal models of nerve injury-induced pain has contributed significantly to our understanding of mechanisms that underlie neuropathic pain (including spontaneous pain, as well as allodynia and hyperalgesia), and to the development of novel pharmacotherapies. Unfortunately, many patients are still not adequately treated and existing drugs have unacceptable side effects. New approaches to the management of ongoing pain, in general and neuropathic pain in particular are clearly needed.

Pain is comprised of two dimensions – a sensory component, referring to the localization, identification and intensity of the noxious stimulus; and an affective component, namely the emotional aspect of the pain experience. The sensory component is mediated by circuits in the primary somatosensory cortex (S1). In contrast, the rostral anterior cingulate cortex (rACC), insular cortex and amygdala are strongly implicated in processing of the affective component of the pain experience. In particular, patients who have damage to the ACC or cingulum bundle, which is a major afferent pathway interconnecting the frontal cortex with the ACC, typically find painful stimuli no longer bothersome (Foltz and White, 1962). One view of the contribution of

pain-related activity in the ACC, is that the aversiveness that underlies this activity serves as a teaching signal (punishment) that shapes subsequent behavior, for example, avoidance or escape from the precipitating stimulus. However, when aversiveness persists, in some instances even when the injury has healed, pain loses this value. In this setting the persistence of pain, i.e. chronic pain, is maladaptive. In our opinion, neuropathic pain, namely pain that arises from injury to the nervous system itself, should be considered a disease of the nervous system. Along with a number of sensory abnormalities, one of the major clinical manifestations of the disease of neuropathic pain is ongoing pain.

The results from clinical studies have important parallels in preclinical studies. For example, lesions of the rACC in rodents significantly reduce conditioned place aversion (CPA), a behavioral measure of an animal's dislike or fear of a noxious stimulus (Johansen et al., 2001). This contribution of the ACC appears to be specifically relevant in a pain context. Thus, Johansen and colleagues showed that bilateral rACC lesions reduced CPA, in response to a noxious stimulus to the hindpaw, but not to systemic U69,593, a kappa opioid receptor agonist that produce significant dysphoria. In other words, although rACC mediates the aversiveness of noxious stimuli, this is not the case for all aversive stimuli. In this regard, the ACC differs considerably from the contribution of the amygdala, which has also been implicated in similar forms of aversion processing. The amygdala mediates avoidance behavior to all aversive stimuli. It appears therefore, that, the rACC is a critical brain region that mediates specifically an organism's ability to recognize a painful stimulus as aversive.

Functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies have shown that the unpleasantness of a noxious heat stimulus applied to the arm correlates highly with activity within the rACC. In one particularly relevant study, subjects who were hypnotized to perceive an intense heat stimulus as more or less unpleasant had an increase or decrease in rACC activity, respectively, but no change in the activity in S1 (Rainville et al., 1997). In other words, the sensory-discriminative processing of the stimulus was not altered by the hypnotic suggestion. Likewise, withdrawal thresholds to a noxious stimulus, which reflect a spinally mediated event, are not altered in nerve-injured rats that have undergone ACC lesions (Johansen et al., 2001; Qu et al., 2011). In contrast, formalin-induced conditioned place aversion, a supraspinally-mediated event that is presumed to be a manifestation of the aversive quality of the stimulus, is blocked in rats with ACC lesions, implying that the ACC contributes to the motivational state that induces an animal to avoid or relieve a painful stimulus. This motivational state likely requires the processing of the unpleasantness of the stimulus itself (Donahue et al., 2001). As noted above, and consistent with the contribution of the ACC, destruction of the rACC or cingulum bundle provides significant pain relief in 50-70% of patients. Taken together these findings demonstrate that targeting cortical areas involved in processing of the pain experience is a logical therapeutic target for managing chronic pain.

GABAergic mechanisms in the ACC

There is now considerable evidence that loss of GABAergic inhibition within the ACC is critical to maintenance of the chronic pain state produced by peripheral nerve injury. For example, Narita et. al. showed that peripheral nerve injury induced upregulation of the GABA transporters (GAT) 2 and 3, which are responsible for removing GABA from the synaptic cleft, in astrocytes of the rACC (Narita et al., 2011). As expected, the levels of GABA in the rACC decreased significantly following nerve injury. Interestingly, restoration of GABA levels by intra-ACC injection of GAT-3 inhibitor reversed the sleep deficit that is assumed to be a behavioral correlate of pain, associated with the nerve injury. Consistent with these findings, other studies reported that injection of $GABA_A$ or $GABA_B$ receptor agonists into the rACC of nerve-injured mice reduce pain. In these studies, the endpoint was either a reduction in noxious stimulus provoked CPA (LaGraize and Fuchs, 2007) or fear conditioning to a painful foot-shock (Tang et al., 2005). Taken together these studies suggest that augmenting GABA-mediated signaling in the rACC is a potential therapeutic strategy for alleviating the tonic state of pain aversiveness associated with neuropathic pain.

An alternative to pharmacotherapy is to provide a continuous and local delivery of inhibitory neurotransmitters. This approach not only more closely recapitulates the endogenous condition, but may also avoid many of the adverse side effects associated with systemic drug administration. Local drug administration via an implanted pump is feasible, but even in this situation the therapeutic agent is not a part of an integrated circuit and has to be replenished from an exogenous source. The ideal

pharmacotherapy would provide a potentially sustainable source of the inhibitory neurotransmitter within cortical circuits, rather than by flooding the cortex.

Most, if not all, GABAergic inhibitory interneurons in the cerebral cortex derive from two regions of the ventral telencephalon: the caudal (CGE) and medial ganglionic eminence (MGE) (Wonders and Anderson, 2006). During early development, MGE progenitor cells migrate dorsally into the cerebral cortex, where they differentiate into different morphological subtypes of interneurons. Seventy percent of these interneurons co-express either somatostatin or parvalbumin. Remarkably, when transplanted into adult brain, embryonic MGE cells show robust migration and differentiation into GABAergic interneurons. Furthermore, MGE-derived cells integrate into host neuronal circuitry and can influence synaptic transmission. In fact, seizure reduction in a mouse epilepsy model could be attributed to a selective increase of $GABA_A$-mediated inhibition of neurons in the transplanted cortical tissue. In another study, Calcagnotto and colleagues (Calcagnotto et al., 2010a; Calcagnotto et al., 2010b) demonstrated that MGE-derived interneurons integrate into host hippocampal circuitry, even when the environment is pathological, created by a chemical ablation of hippocampal inhibitory interneurons. Most importantly, by replacing the ablated GABAergic interneurons, the MGE transplants restored normal hippocampal synaptic inhibition. Peripheral nerve injury also leads to a decrease in GABAergic tone within the dorsal horn of the spinal cord, which contributes to a hyperexcitable state that is a critical contributor to the mechanical allodynia and chronification of the neuropathic pain state. Finally, although MGE cells are not the normal origin of spinal cord GABAergic interneurons, studies in

our laboratory found that transplanting MGE cells into the spinal cord of nerve-injured mice was very effective at reversing the mechanical hypersensitivity caused by peripheral nerve injury (Braz et al., 2012).

Given the remarkable ability of MGE cell transplant to overcome loss of inhibition, here we tested the hypothesis that chronic replacement of GABA by using transplanting MGE cells into the rACC can reverse the affective component of pain (pain aversiveness) that is normally regulated by this brain region. To determine whether the animal is experiencing tonic pain aversiveness, we took advantage of the analgesia-induced conditioned place preference (CPP) paradigm. In this behavioral test, animals learn to associate one side of a two-chambered apparatus with administration of the analgesic (i.e. pain relieving) drug effective against neuropathic pain, namely gabapentin.

Importantly, gabapentin does not inherently have rewarding properties in uninjured animals. However, in the setting of nerve injury, we demonstrated that mice increase their preference for the gabapentin-paired side, indicating that they experienced pain relief from the drug. In this way, we can determine if an animal is experiencing a tonic pain aversiveness caused by the nerve injury, and can also whether a different, non-rewarding treatment (viz., MGE cell transplants into the rACC) can alleviate the pain. If the MGE cell transplants, in fact, reduce tonic pain aversiveness, then preference for the gabapentin-paired side of the CPP apparatus should be reduced, i.e. the “need” for the gabapentin is reduced. This finding would argue strongly that reduced GABA

signaling is indeed a major contributor to maintenance of the affective component of the neuropathic pain state.

Results

Previous studies in the rat demonstrated that lesions of the rostral anterior cingulate cortex (rACC) do not affect the sensory discriminative aspects of the pain experience, but can alleviate its aversive quality. To determine if this relationship holds true in mice, our first experiments repeated those initially performed in the rat. Specifically, we studied the effect of rACC lesions in an acute and a chronic inflammatory pain model. In these studies, we tested the animal's responsiveness to a noxious heat stimulus applied to the hindpaw and to an intraplantar injection of dilute formalin (1%). The studies were performed in control mice and in mice in which the rACC was ablated by an excitotoxic lesion.

Baseline thermal thresholds were determined prior to ablating the rACC and then again 6 days after the rACC lesion (**Figure 1a & b**; N = 13); control animals underwent sham surgery (control, N = 6), in which only the scalp was exposed. As predicted, compared to baseline responses, we found no difference in thermal thresholds control and lesion groups (**Figure 1c**; One-way ANOVA, Dunnett's multiple comparisons test, n.s.). In the formalin test, lesion (N = 12) and control (N = 9) animals received an intraplantar injection of 20µl 1% formalin into the left hindpaw and nocifensive behaviors (paw flinching and licking) were video recorded for 45min. Scoring of nocifensive behavior was binned into 5 minute intervals. Again, we found no difference in nocifensive

behavior at any of the time points between the lesion and control groups (**Figure 1d**; 2-way ANOVA, Sidak's multiple comparisons test, n.s.).

In another set of experiments we studied the effects of rACC lesions on the mechanical allodynia produced in in the sciatic nerve injury (SNI) model of neuropathic pain. One week after SNI, mechanical thresholds were assessed with von Frey filaments, so as to determine the magnitude of the mechanical hypersensitivity of the ipsilateral, injured paw. One day later, animals underwent rACC lesion or sham surgery. After a 6 day recovery period, we retested mechanical thresholds of the ipsilateral and contralateral paws. Again we found was no difference between the magnitude of mechanical allodynia in the lesion (N = 8) and control (N = 7) groups (**Figure 1e**; RM 2-way ANOVA, Sidak's multiple comparisons test, n.s.). The mechanical threshold of the uninjured paw also was unaffected by rACC lesions (**Figure 1f**; RM 2-way ANOVA, Sidak's multiple comparisons test, n.s.). Taken together, these data indicate that as in the rat, rACC lesions do not reduce the sensory discriminative aspect of the pain experience.

Next we examined the contribution of the rACC to the aversive aspect of the pain experience. ACC hyperexcitability, which is presumed to underlie the aversiveness associated with nerve injury, has been demonstrated in both patients using fMRI (Hashmi et al., 2013) and in preclinical models of neuropathic pain using in vitro electrophysiological studies (Zhuo, 2014). Whether the increased excitability arises from increased excitatory neurotransmitter signaling or from a decrease in inhibitory

neurotransmitter tone is not clear. For example, Zhuo and colleagues reported that peripheral nerve injury induces the expression of protein kinase M, ζ (PKMζ) in the ACC, and that this increase is responsible for the maintenance of ACC hyperexcitability. Pharmacological blockade of PKMζ in the ACC eliminated the hyperexcitability, as well as behavioral sensitization (Li et al., 2010). Of particular interest are studies of Narita and colleagues who reported that the GABA transporter 3 (GAT3) protein that is responsible for removing GABA from the synaptic cleft, is upregulated in ACC astrocytes following peripheral nerve injury. The end result is a decrease in GABAergic tone within the ACC. In the same report the authors found less evoked GABA release from ACC synapses following peripheral nerve injury.

Here we first investigated other features of GABAergic signaling in the setting of nerve injury. In these experiments, we performed quantitative PCR (qPCR) on rACC tissue dissected from sham-operated (N = 4) and SNI-operated mice at 14 (N = 4) and 30 days (N = 4) after injury. We assessed the mRNA levels of GAD65, GAD67, GAT3, GABARa1, GABAB-R1, and VGAT (**Figure 2c**). GABAB-R1 mRNA increased by 26% at 14d post-SNI, but returned to baseline levels by day 30 (**Figure 2b**; One way ANOVA, Tukey's multiple comparison test, $p < 0.01$). VGAT mRNA in the rACC showed no change from sham animals at 14d post-SNI, but then decreased by 49% by 30d post-SNI (**Figure 2a**; One way ANOVA, Tukey's multiple comparisons test, $p < 0.01$). The change in VGAT is particularly interesting as Narita et. al., using microdialysis, found that evoked release of GABA is diminished in the ACC of nerve-injured rats (Narita et al., 2011). A decrease in VGAT suggests that GABA is less efficiently loaded

into synaptic vesicles, which could manifest as a decrease in the evoked release of GABA and reduced GABAergic tone. The result of these changes would be an increased excitability of the ACC. Taken together it appears that increased ACC excitability results from a combination of increased excitatory mechanisms as well as a decrease in inhibitory tone.

As noted above, transplants of progenitor cells from the embryonic cortex that are destined to become GABAergic inhibitory interneurons mature, integrate and can overcome loss of inhibition. Successful transplants have been reported for cerebral cortex and in the spinal cord. In the present series of studies, we tested the hypothesis that transplanting MGE cells into the rACC of mice with nerve-injury can also restore GABAergic inhibitory tone to the rACC and therefore reduce the aversiveness of ongoing pain caused by nerve injury.

In these experiments, we dissected MGE cells from GAD67-GFP embryos. The GFP provides a critical reporter that allows for monitoring of the efficiency of the transplanted cells in the host. Our studies were performed in mice in which we created a model of chemotherapy induced neuropathic pain, which is also manifest by signficant mechanical hypersensitivity (in this case, of both hindpaws). We transplanted MGE cells into the ACC one week after paclitaxel administration. **Figure 3a & b** shows examples of GFP+ MGE cells that were transplanted into the ACC 30d earlier. At 30 days, we found that the cells migrate up to 1.7mm total (i.e. 850μm in the rostral and caudal directions) from the injection site. To establish that the transplanted indeed

developed into mature neurons and expressed markers of cortical interneurons we examined the neurochemistry of these cells. In these studies, we immunostained the cells for a pan neuronal marker (NeuN) and GABA and for somatostain (SST) and parvalbumin (PV), two of most common subpopulations of GABAergic interneurons that are derived from the MGE. We also immunostained the cells for non-neuronal markers (astrocyte using a GFAP antibody), and microglia (using an antibody against Iba1. **Figure 3c** illustrates GFP immunoreactive cells that co-express SST. Surprisingly, the majority of GFP+ cells did not label for PV (**Figure 3d**). **Figure 3e** provides a summary of the results, where SST/GFP (31%), PV/GFP (2%), GABA/GFP (60%), and NeuN/GFP (84%). No GFP+ cells labeled for glial markers.

Having established that the cells survive transplant into the ACC and express markers of GABAergic neurons, we next asked whether the MGE cells exhibit the intrinsic properties of typical SST+ and PV+ inhibitory neurons. In these studies, we performed whole-cell patch clamp recordings from GFP+ cells in 400μm thick coronal sections of the ACC, 30d after transplant. In current-clamp mode, we were able to identify all firing patterns typically associated with cortical inhibitory interneurons (Kawaguchi and Kondo, 2002). Most prominent are the fast-spiking (Rm < 350, ISI < 1.3) and latent-spiking phenotypes characteristic of PV+ cortical interneurons, (**Fig, 4a**; N = 8). We also recorded non-fast spiking GFP+ cells (**Fig. 4b**; N = 10), which is characteristic of the SST+ population of interneurons.

Having established the intrinsic properties of the transplanted neurons, we next performed blind, paired recordings from GFP+ neurons and nearby host pyramidal neurons. The GFP+ and neighboring host pyramidal neurons were held in current and voltage-clamp (-70mV), respectively (the latter with a high chloride internal-pipette solution). **Figure 4c (top right panel)** shows that during blind paired-recordings, current injection into the GFP+ neurons in Layer II/II elicited a series of spikes in the interneurons and also resulted in a chloride current in 3/17 pyramidal neurons (**Figure 4c, bottom right panel**), establishing that at least some of the transplanted MGE neurons were functionally connected to host pyramidal neurons. To determine whether these currents were mediated by GABA, we applied 10μM gabazine (a $GABA_A$-receptor antagonist) to the bath. Figure 8C shows that gabazine, indeed blocked the chloride current in these pyramidal neuron (**Fig. 4c**, right panel).

In another approach to identify functionally connected MGE and host neurons, we studied acutely dissected brain slices (400μm) from animals (N = 3) in which we had transplanted MGE neurons derived from the I12b-ChR2+ mouse line. In these mice, Channelrhodopsin2 is expressed under a GABAergic-neuron specific promotor in the MGE cells, which made it possible to activate simultaneously a population of MGE neurons. In these experiments we applied a 20ms light pulse (460nm) while holding pyramidal neurons in voltage-clamp (+10mV) and observed evoked inhibitory postsynaptic currents (IPSCs; **Figure 5a, left panel schematic**) in 12/19 host pyramidal neurons (mean (±SEM) IPSC amplitude: 106±22μA). In all neurons the MGE-evoked IPSCs were blocked by bath application of the $GABA_A$-R antagonist, bicuculline (10μM;

mean ±SEM drop value: 75±3%; N = 10; **Figure 5b,** top trace; baseline vs. bicuculline, p = 0.0012; Freidman test with Dunn's multiple comparison correction) but not by a $GABA_BR$ antagonist, CGP55845 (2.0 μM; N = 7; **Figure 5d and e,** middle trace). Importantly, the amplitude of the optogenetically-evoked IPSCs returned to baseline levels after a 10min wash, which remove the bicuculline (**Figure 5b**, top trace; bicuculline vs. wash, p = 0.014; Freidman test with Dunn's multiple comparison correction). We conclude that the MGE neurons release GABA, and that the GABA signals through the $GABA_A$-receptor. The lack of effect of the $GABA_B$ receptor antagonist is consistent with the very limited to no expression of $GABA_B$ receptors in cerebral cortex.

Finally, we asked whether activation of MGE neurons could functionally inhibit current evoked action potentials in host pyramidal neurons. Pyramidal neurons were injected with current so as to induce action potential firing, and this was followed by a 100ms flash of blue light. As illustrated in **Figure 5f**, optogenetic activation of MGE neurons with blue light immediately inhibited action potential firing in the host pyramidal neurons, but only for the duration of the light exposure. Taken together, these data provide strong evidence that the transplanted MGE neurons successfully integrate into host cortical circuitry. MGE neurons form GABAergic synapses and mainly act to shut off action potential firing in pyramidal neurons via the $GABA_AR$. Based on the time-locked inhibition, we also suggest that the inhibition involves a circuit based synaptic inhibition, rather than pump-like diffusion of GABA from the transplanted cells.

Having established that the MGE cells form functional output synapses onto host pyramidal neurons, we also sought correlate anatomical evidence, as to the origin of the inputs to the transplanted neurons. In these studies, we took advantage of the EnVA-TVA (Callaway and Luo, 2015) system in which an avian-pseudotyped rabies virus expressing EnVA-RFP-ΔG is injected into the rACC 30 days post-MGE transplant. In this case, the MGE cells were taken from a I12b-Cre x ROSA-tdTomato mouse that had been crossed with a lox-STOP-lox-TVA_RG mouse, which induces Cre-dependent expression of the avian receptor TVA. Expression of the TVA receptor selectively enables the EnVA-rabies virus to enter these cells. However, because the MGE neuron also carries the rabies glycoprotein (RG) construct, the virus can only move retrogradely by one synapse. As the presynaptic neuron does not express protein G, the virus is trapped in this immediately presynaptic neuron, allowing identification of neurons that are monosynaptically connected, presynaptic partners of the MGE cells.

Figure 6a shows an example of the MGE injection site in which uninfected MGE neurons only express RFP. Starter cells, which took up the rabies virus, are yellow indicating co-expression of both RFP and GFP (from the virus; yellow arrowheads). Monosynaptically connected, presynaptic partners of the MGE neurons, are labeled with GFP only (white arrowheads). Based on analysis of 4 different mice, we observed consistent labeling of long distance monosynaptic presynaptic partners of the MGE cells, including GFP+ neurons in the ipsilateral (and less frequently contralateral) pACC (**Figure 6b**), the nucleus of the horizontal limb of the diagonal band, medial thalamic nuclei, lateral hypothalamus (LH), medial amygdala (AMYG, **Figure 6c**) and medial

septum (MS). These results compare very well with previous retrograde tracing studies that examined input to the cingulate cortex (Horikawa et al., 1988). Of course, those studies could not define the neuronal target of the distant projections to the ACC. Our results provide convincing anatomical evidence that local as well as distant host neurons engage the transplanted MGE cells. Taken together with the electrophysiology analysis, we conclude that MGE cells successfully integrate into both local and long-distance ACC host circuitry. We believe that this is the first demonstration that long-distance afferents that derive from from different brain regions innervate transplanted MGE neurons.

Having characterized the MGE cells and established that they mature into functional GABAergic interneurons when transplanted into the ACC of adult mice, we turned our attention to the behavioral consequence of this novel approach to re-establishing inhibitory control in the ACC. Here we tested our hypothesis that transplanting MGE cells into the ACC of injured mice can restore GABAergic inhibitory tone to the rACC and thereby reduce the pain aversiveness characteristic of this neuropathic pain model. In these experiments, we used the gabapentin-induced conditioned place preference paradigm (GP-CPP) described in Chapter 1. As noted above, gabapentin is not inherently rewarding in uninjured animals, i.e., it does not induce any preference for the drug in uninjured animals, but does have an analgesic (pain-relieving) action in injured animals. In other words, nerve injured mice that are mechanically hypersensitive show a change in preference for the GP-paired chamber (indicative of pain relief, i.e. negative reinforcement; **CPP Score** = test day preference- pretest preference).

In these experiments we used the paclitaxel-induced chemotherapy-model of neuropathic pain as there is a robust whole-body hypersensitivity phenotype. We reasoned that this robust pain phenotype would increase our ability to demonstrate a preference for a drug that reduces aversiveness of the condition. In these experiments, we tested the hypothesis that paclitaxel-treated animals would show a preference for the GP-paired chamber. However, paclitaxel-treated animals that received MGE transplants in the ACC would not show a preference for the GP-paired chamber because the transplanted cells restored inhibitory control in the ACC and reduced the pain aversiveness produced by taxol treatment.

As noted above, MGE cells migrate considerable distances from the injection site (covering a total distance of 1700μm), but importantly, they rarely moved laterally. As a result, the GFP+ neurons ended up in multiple brain regions, including the rostral ACC (**rACC**, stereotaxic coordinates: +2.46 to +1.18mm from bregma), posterior ACC (**pACC**, stereotaxic coordinates: +1.10 to +0.20mm from bregma), secondary motor cortex (**M2**) and the prelimbic cortex (**PrL**). Upon careful analysis of the cell distribution, it became clear that there were two major groups of animals whose behavior correlated best with cell distributions in the rostral and posterior parts of the ACC. Control animals (injected with DMEM only; N = 18) showed an average CPP score of 162.1 ± 29.30 sec, indicating that gabapentin was pain relieving (**Figure 7a**). As predicted, transplanting MGE cells in the rACC was pain relieving because MGE-transplanted animals did not show a change in preference for gabapentin when the GFP+ cells were concentrated to

the rACC and < 150 cells were counted in the pACC (N = 24; CPP Score = 44.38 ± 24.78 sec). However, animals that had MGE neurons in both the rACC and pACC (>150 cells) showed a large preference for the GP-paired chamber (N = 7; CPP Score = 92 ± 55.24 sec). Johansen and colleagues previously reported that the rACC and pACC are not functionally homogenous brain regions and that lesions of each have difference effects on behavior (Johansen et al., 2001). Considering this, our result was unexpected and suggests that substantial MGE-mediated inhibition of the pACC can counteract the analgesic effect of increasing inhibitory tone in the rACC (**Figure 7a**; One way ANOVA – Kruskal-Wallis test with Dunn's multiple comparison correction; <150 vs. >150 cells in pACC, $p < 0.01$; DMEM vs. >150 cells in pACC, n.s.). Importantly, this counteracting effect was limited to the posterior ACC. The number of cells in the PrL and M2 regions did not correlate with the CPP Score of MGE-transplanted mice (**Figure 7b and c**; One-way ANOVA, Kruskal-Wallis test Dunn's multiple comparison correction, n.s.).

We next asked whether comparable results could be produced by excitotoxic lesions of the rACC and pACC. Therefore, in another group of studies we ablated the rACC only, or the rACC + pACC one week after paclitaxel administration, then conducted the GP-CPP paradigm. Control animals (N = 18) that received no lesion showed an average CPP score of 100 ± 23.24 sec (**Figure 8**). Animals that had lesions only in the rACC (N = 5) did not show a preference for gabapentin (CPP score = -23.97 ± 27.65 sec). Interestingly, just as in the MGE transplant experiments, animals that had lesions of the rACC + pACC (N = 12) showed a preference for gabapentin once again, with an average CPP score of 98.33 ± 29.58 sec, indicating that pACC lesions counteract the

analgesic effect of rACC lesions. This result significantly strengthens the conclusion that inactivation of the rACC, whether it by via increased inhibition or by excitotoxic ablation, is pain relieving and that the pain relief, unexpectedly, can be reversed by inactivation of the pACC. To our knowledge, this is the first description of a direct interplay between circuits that integrate rACC and pACC function, the consequence of which is a measurable behavioral output in a preclinical model of neuropathic pain. Our findings underscore the complexity of the cortical circuits that regulate the affective component of the pain experience.

Discussion

The anterior cingulate cortex is a limbic brain region that in humans has been strongly implicated in the affective/emotional component of the pain experience. Here we show that rACC serves the same function in the mouse and, as in the rat, appears not to be involved in the sensory-discriminative aspects of the pain experience. For example, rACC lesions had no effect on thermal thresholds, formalin licking, or the development of mechanical allodynia in nerve-injured animals. This segregation of function was also observed after MGE transplants, where we found that MGE cell-mediated inhibition of the rACC had no effect on mechanical allodynia in the paclitaxel model of neuropathic pain. On the other hand, MGE cell transplants into the rACC showed remarkable analgesic efficacy, i.e. they produced significant pain relief in the CPP test. The transplants reversed the aversiveness of ongoing pain produced following paclitaxel. Unexpectedly, as a result of the migration of the transplanted cells from the injection site, we uncovered a very different contribution of inhibiting the activity of pACC

neurons. Most surprisingly, when the injection resulted in large numbers of MGE cells surviving in both the rACC and pACC, the pain relieving effect of rACC-inhibition was lost. A comparable result was produced by excitotoxic lesions of both the rACC and pACC in injured animals. Specifically, the affective component of the pain experience reappeared and was manifest as a reinstatement of the preference for gabapentin in the paclitaxel model.

Properties of the rACC and pACC:

Previous studies reported that only the rACC, not the pACC, has a primary role in the affective component of pain perception. Thus, in rats, excitotoxic lesions of the rACC blocked the behavioral manifestation of aversion to intraplantar formalin, whereas lesions of the pACC had no effect (Johansen et al., 2001). rACC lesions also blocked the preference for intrathecal clonidine or RVM lidocaine in rats with nerve injury, indicating that the aversiveness of ongoing pain is lost when the rACC is ablated (Qu et al., 2011). Importantly, rACC lesions did not block the ability of the animal to show a preference for cocaine, a rewarding stimulus that has no analgesic properties. That result demonstrates that the lesion did not produce a generalized inability of the animal to form an association of a particular side of the CPP apparatus with appetitive stimuli (Qu et al., 2011). Unexpectedly, we found that animals with a combined lesion of the rACC and pACC showed reversal of the analgesic (anti-aversive) effect observed after rACC lesions. Thus, rACC-pACC lesioned animals had the same level of preference for gabapentin as did animals without a lesion. This finding mirrored what we observed after MGE transplants. That is, animals in which the transplanted cells were focused

largely in the rACC, and not the pACC, lost the pain aversiveness associated with paclitaxel-induced neuropathy. However, animals with MGE cells in both the rACC and pACC had a large preference for gabapentin, comparable to that of DMEM-injected animals.

Intracingulate connections

There is anatomical evidence for dense intra-cingulate projections in both the rodent and monkey (Fisk and Wyss, 1999; Jones et al., 2005). Injections of anterograde tracer along the axis of the ACC result produce different patterns of axon labeling. For example, tracer injections in the rostralmost portion of the ventral pACC result in dense axon labeling in the dorsal pACC and less dense in the rACC. Tracer injections into the caudal-most half of the pACC result in dense labeling in the rostral half of the pACC, and dense labeling of the retrosplenal cortex (Rsg). However, injections into the rACC show almost no cingulate projections, whereas injections into the rostralmost part of the dorsal pACC, the primary location of our pACC lesions, produce particularly dense axonal labeling in the rACC and caudal pACC (Jones et al., 2005). Despite the considerable anatomical information there is very limited information about the functional significance of the interconnections.

Based on our rACC/pACC MGE findings, is is possible that in the absence of injury, the pACC exerts a tight inhibitory control over the rACC. GABAergic inactivation of the pACC would, therefore, result in disinhibition of the rACC, increasing rACC activity, which we and others have demonstrated contributes to an increase in the aversiveness

of the painful experience. What is difficult to explain is the consequence of transplants that we presume should concurrently inhibit both the pACC and rACC. Clearly there must be some threshold of inhibition of the rACC that is required for its “pro-aversive” function to be blocked. Presumably reinstatement of the tonic state of pain aversiveness normally generated by rACC activity, would require that the disinhibition of the pACC input is strong enough to override the MGE-mediated inhibition in the rACC. Alternatively, and strongly supported by the rACC/pACC lesion results, we suggest that pACC inactivation results from disinhibition of a yet to be determined brain region that also contributes to pain aversiveness, particularly when there is concurrent inhibition of the pACC and rACC. In this setting, i.e, even when the function of the rACC is lost, it would still be possible to induce a preference for gabapentin in nerve-injured animals. Below we address three brain regions that may contribute to pain aversiveness in addition to, or in concert with, the rACC.

Contribution of the amygdala

The amygdala is of particular importance for assigning emotional valence, primarily aversiveness, to stimuli in the environment and events. The amygdala receives purely nociceptive input from lamina I of the spinal cord that is relayed either via the parabrachial nucleus (PB) to the central nucleus of the amygdala (CeA) or through the nociceptive thalamus, which send its input to the lateral nucleus of the amygdala (LA). The input-output circuit within the amygdala is well established, with neurons of the LA targeting the BLA, which in turn projects to the CeA. Although the CeA is the primary output nucleus of the amygdala, the BLA is the main source of amygdala projections to

the rACC. The CeA sends inputs to brainstem regions involved in descending inhibitory mechanisms, as well as to other brain regions regulating emotional responses and affective states.

In preclinical models of arthritic (Neugebauer and Li, 2003), visceral (Han and Neugebauer, 2004), and neuropathic pain (Ikeda et al., 2007) PB-CeA and BLA-CeA synapses are potentiated. Pharmacological inactivation of the CeA decreases spinal withdrawal reflexes as well as brain-dependent pain behaviors, including ultrasonic vocalizations which are increased in models of arthritic pain (Han and Neugebauer, 2005). As for rACC lesions, CeA lesions abolish conditioned place aversion to hind paw injection of formalin, but have no effect on spontaneous nocifensive behaviors (Helmstetter and Bellgowan, 1993; Tanimoto et al., 2003), which are presumed to be a manifestation of nociceptive transmission. Furthermore, microinjection of a $GABA_A$ receptor agonist into the CeA attenuates avoidance behavior and reverses mechanical allodynia in nerve injured rats (Pedersen et al., 2007). Conversely, activation of the amygdala can also be anti-aversive (Helmstetter, 1992). These studies suggest that the CeA is a possible locus where reinstatement of pain aversiveness arises when there is concurrent ablation of the pACC and rACC.

Although the amygdala clearly has a defined role in assigning negative valence to painful stimuli, activity of the amygdala is thought to be more involved in the expression of conditioned reactions to aversive stimuli, rather than mediating only the percept of aversiveness (Watkins et al., 1993). This distinction differentiates the amygdala from

the rACC. The rACC functions to increase the salience of painful stimuli in particular, not to all aversive stimuli, and rACC activation acts as a teaching/motivational signal that induces a conditioned response, to avoid or escape. We believe that the rACC is the ACC subregion that is required for this teaching signal to occur. For example, in the case of avoidance behaviors, rACC lesions or glutamate receptor blockade abolish the formalin-induced conditioned place avoidance (Johansen and Fields, 2004; Johansen et al., 2001). Because the amygdala shares reciprocal projections with the rACC, lesions of the rACC abolish the rACC-to-amygdala teaching signal that is responsible for enhancing the salience of painful stimuli. It appears that, under these circumstances, the amygdala does not receive sufficient information to induce avoidance behavior. However, based on our findings, we suggest that when the contribution of the pACC is concurrently lost, then feedback activity from the ACC is absent. As a result, all regions that share reciprocal connections with the ACC become unregulated. We hypothesize that these unregulated regions must be capable of mediating pain perception, and must also project to the amygdala. Because these regions now receive aberrant signals, they could send altered signals to the amygdala and thus result in amygdala-mediated avoidance. In the context of our experiments, this aberrant signaling could reestablish a state of tonic pain aversiveness, which in our assay, manifests as a preference for gabapentin.

Contribution of the mediodorsal thalamus

Another candidate brain region that may be implicated in the pro-algesic (pro-aversive) behaviors observed when both the rACC and pACC are lesioned is the mediodorsal

thalamus (MD). The MD has strong recriprocal projections with the amygdala and pACC. The MD also receives direct projections from nociceptive neurons in lamina I of the spinal cord, and a convergent input from the parabrachial nucleus, a major source of inputs to emotional processing areas of the brain that drive pain aversion. Conceivably, the MD functions as a node of integration that balances activity within all of these brain regions in response to noxious stimuli. In rACC-pACC lesioned animals, a lack of feedback from the pACC to MD circuits would result in a significant alteration of nociceptive signals that are relayed to other sites that process affective information, such as the insular cortex.

Contribution of the anterior insula

Imaging studies in humans show increased activity in the rostral ACC and insular cortex of chronic lower-back pain patients (Baliki et al., 2006). Patients with insular-opercular strokes report a somesthetic-limbic disconnect in which noxious stimuli are no longer perceived as aversive (Biemond, 1956). Furthermore, peripheral nerve injury is associated with decreased brain volumes in both the ACC and insula in rats (Seminowicz et al., 2009). These data taken together suggest that the ACC and AI are concurrently influenced by peripheral nerve damage. Interestingly, lesions of the insula have dual effects on nociception. Inactivation of the insular cortex has been shown to be antinociceptive, pronociceptive, or have no effect (Coffeen et al., 2011; Jasmin et al., 2003; Shi and Davis, 1999). Anatomical studies show that anterior insula has reciprocal connections with the rACC/pACC, amygdala, and other primary sensory cortices (olfactory, gustatory, & visceral somatosensation), making it a prime node for the

assignment of emotional valence to different sensory modalities. It follows that lesioning both the pACC and rACC could result in an imbalance of aversive inputs that are channeled into the AI from the medial thalamus and amygdala, both of which lack the feedback control from the ACC.

Hyperexcitability of the ACC

Many studies, both in humans and animal models, demonstrated that the ACC becomes hyperexcitable after peripheral nerve injury (Baliki et al., 2006; Hashmi et al., 2013; Zhuo, 2014). Min Zhuo and colleagues demonstrated long-term potentiation at ACC synapses in injured animals, and later showed that intra-ACC pharmacological blockade of this LTP induction is rewarding in nerve-injured mice (Li et al., 2010). In our opinion, a major contributor to the hyperexcitability is nerve injury-induced loss of local inhibitory tone. In our study, we looked for signs of altered GABAergic control in nerve injured animals. Using qPCR we found found that VGAT mRNA was decreased by 49% in SNI animals. This follows nicely with the work of Narita and colleagues who reported injured animals have less evoked GABA release at ACC synapses (Narita et al., 2011). Furthermore, nerve injury reduces the number of ACC inhibitory synapses onto pyramidal neurons, as well as excitatory connections onto inhibitory neurons (Blom et. al. 2014).

Classification of the MGE transplants

We found that MGE cells successfully mature and integrate into the circuitry of the cortex of adult mice with neuropathic pain. As in previous transplant studies (Alvarez-

Dolado et al., 2006; Baraban et al., 2009), the MGE cells differentiated into different subtypes of inhibitory interneurons. Thirty-one percent of the cells were positive for somatostatin, a slightly lower percentage than that reported in earlier studies (40-43%; (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Braz et al., 2012). The earlier studies examined transplants into adult spinal cord (Braz et al., 2012) or in the cortex of postnatal (P2-P4) animals, some of which experienced cortical seizures (Baraban et al., 2009). Conceivably the lower recovery rate in our analysis reflects the fact that the cells were transplanted into the adult cortex of paclitaxel-treated animals.

We also confirmed by patch electrophysiology that a portion of the MGE cells displayed characteristic non-fast spiking firing pattern, consistent with the somatostatin population (Alvarez-Dolado et al., 2006). Likewise, although we did not detect parvalbumin-immunoreactive MGE neurons, we did record some MGE cells with intrinsic firing patterns that are characteristic of cortical parvalbumin-positive GABAergic interneurons (Kawaguchi and Kondo, 2002). These include both fast-spiking and latent fast-spiking neurons. We appreciate that some of the transplant may have included cells that derived from the LGE or CGE populations of inhibitory interneurons. Regardless of the subtype of transplanted neurons, it is clear that the functional consequence of transplantation resulted from a GABAergic inhibition. Thus, MGE activation was sufficient to block current injection-evoked action potentials in host pyramidal neurons, in a $GABA_AR$-mediated manner.

MGE circuit integration

Using a viral tracing approach we found anatomical evidence that MGE transplants receive inputs from local and distant host neurons. However, although both the MGE and viral injections targeted and indeed were concentrated in the rACC in all 4 mice, it is likely that both the virus (due to diffusion) and cells (due to their migration) extended to more rostral or caudal regions near the injection site. As a result it is almost certain that the retrogradely labeled presynaptic partners of MGE neurons included areas that project to both the rACC and pACC. Using the EnVA-TVA strategy, we found labeled presynaptic neurons in a variety of regions, including several nociceptive thalamic, the lateral hypothalamus, amygdala, all of which have been implicated the affective aspects of the pain perception.

Our findings are consistent with previous anatomical tracing studies in the rodent (Gaykema et al., 1990; Saper, 1985). The medial and intralaminar thalamic nuclei, LH and amygdala all contain nociresponsive neurons (Craig and Burton, 1981; Dado and Giesler, 1990; Dafny et al., 1996; Dong et al., 1978; Van Groen and Wyss, 1995; Vogt, 2005) and have been implicated in emotion processing. Taken together these studies demonstrate that the MGE neurons in the rACC receive inputs from key distant brain regions that contribute to the activity of the rACC. These data indicate that the MGE cells integrate into critical, long-range neural circuits that contribute to the affective component of the pain experience and therefore are in position to have a significant impact on how nociceptive information is processed in the ACC.

In conclusion, MGE cell transplants into the rACC successfully relieve tonic pain aversiveness in injured mice. Based on these findings, we conclude that: (1) disrupting the activity of the rACC is sufficient for blocking the aversiveness of ongoing pain caused by nerve injury; (2) lesions of the pACC result in a dysregulation of neural activity leaving the ACC which affects a yet to be identified region that can also encode pain aversiveness, even in the absence of the rACC. We suggest that the relevant region is one that has excitatory connections with the amygdala and receives (net) inhibitory inputs from the pACC. Additionally, ACC MGE cell transplants receive relevant neural inputs from brain regions that mediate pain affect. Notably, the pACC (the posterior portion of Broadmann Area 24), which has never in behavioral studies been assumed to interact functionally with the rACC in a pain-specific manner in the rodent, appears to be a critical element in controlling feedback activity that exits the ACC. We conclude that the rostral and posterior portions of Broadmann Area 24 (the rACC and pACC) should be treated as two distinct regions with distinct contributions to pain processing. Future studies should address the local circuitry between the rACC, pACC and distant regions, to better understand the nature of the feedback control exerted by the pACC on nociceptive signals arriving in the ACC, and how these areas affect their output to other brain regions.

Chapter 2

Materials and Methods

Mouse lines:

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California San Francisco. MGE cells were dissected from transgenic mice that express GFP under the control of the GAD67 promoter ($Gad1^{tm1.1Tama}$; (Tamamaki et al., 2003). All transplants were performed on male mice (6–8 weeks old) with the same genetic background as the MGE donors (CD1xC57BL6/J). **Antibodies:**

Chicken anti-GFP (1:2,000; Abcam, Cambridge, UK), mouse anti-PV (1:2,000; Sigma-Aldrich), rabbit anti-GFAP (1:20,000; Dako, Carpinteria, CA, USA), rabbit anti-Iba1 (1:2,000; Wako, Richmond, VA, USA), rabbit anti-SST (1:5,000; Peninsula, T-4103), mouse anti-GABA (1:2,000; Sigma-Aldrich and mouse anti-NeuN (1:2,000; Chemicon, Temecula, CA, USA).

Cell Counts and Quantification

To determine the number of MGE cells that survived the cortical transplantation we counted cells from digitized images of GFP-immunoreactive neurons collected on a Zeiss Axio Image M2 microscope. In each animal we counted all cell bodies in 35 transverse sections (35μm thick) that included the ACC, and estimated percentage 100 x (total GFP+ cells)/(number of cells in the initial transplant). These studies were performed in 31 animals. In a separate group of 3 animals that received MGE

transplants, we assessed the percentage of surviving transplanted cells that expressed a second marker (NeuN, Iba1, GFAP, tdTomato, GABA, PV, or SST), imaged on a Carl Zeiss LSM 700 microscope. These calculations were made from a series that contained all transverse sections (separated by 210 μm) that spanned the cortical areas containing transplanted cells. At least 100 GFP+ MGE cells were analyzed for each marker, in each animal (n = 3). The counts were made 30 days after transplantation. We calculated the percentage of double-labeled neurons (marker+ and GFP+) by dividing the number of double-labeled neurons by the number of single GFP-labeled neurons x 100. Values are given as mean ± standard error (SEM).

Electrophysiology

Mouse lines

For some experiments, MGE cells were dissected from double transgenic I12b-ChR2 mice, which express Channelrhodopsin 2 (ChR2) selectively in the forebrain. These mice were generated by crossing mice that express Cre under the control of the I12b enhancer (I12b-Cre; (Potter et al., 2009)) with mice that express a floxed Channelrhodopsin 2 cDNA under the control of the Rosa26 promoter (Ai32; (Madisen et al., 2012), Jackson Laboratories).

Slice preparation

For these experiments the composition of the dissection solution was (in mM): NMDG 93, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, Glucose 25, sodium ascorbate 5,

Thiourea 2, sodium pyruvate 3, MgSO4-7H2O 10, CaCl2-2H2O 0.5. The composition of the recovery solution was (in mM): NaCl 92, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, Glucose 25, sodium ascorbate 5, Thiourea 2, sodium pyruvate 3, MgSO4-7H2O 2, CaCl2-2H2O 2 (Ting et al, 2014, Tanaka et al, 2008). On the day of the experiment, 8-9 week-old mice, 2-3 weeks following transplantation of embryonic MGE cells were killed with an overdose of Avertin (Sigma-Aldrich, USA). Following transcardial perfusion with 8ml of Dissection solution the brain was quickly removed, mounted in a Vibratome (VT1200, Leica, USA) bath filled with ice-cold dissection solution and 4 transverse or parasagittal sections were cut. The sections were transferred to a glass beaker containing heated (37^{o}C) recording or HEPES-based recovery solution.

Electrophysiological recordings

The sections recovered in heated solution for at least 1 hour before use. Then, the sections were transferred to a recording chamber (Automate Scientific, CA, USA) under an upright fluorescent microscope (Nikon E600FN, Japan) and superfused with recording solution at a rate of 1.0 ml/min. Sections were viewed with a CCD digital camera (Hamamatsu Inc., Japan or DAGE-MTI Inc., USA). Patch pipettes were pulled on a horizontal pipette-puller (Sutter Instrument, USA) from thin-wall, fire-polished, borosilicate glass filaments to yield an impedance of 6-8MΩ. The composition of the pipette solution was (in mM): K-methane sulfonate 140, NaCl 10, $CaCl_2$ 1.0, EGTA 1.0, HEPES 10, Mg-ATP 5.0, NaGTP 0.5 and 5.0 mg/ml of Biocytin (Sigma Aldrich, USA) to allow for subsequent intracellular filling of the recorded cells.

Cells were visualized under near-IR differential interference contrast illumination and approached with a micromanipulator (Sutter Instrument, CA, USA) while monitoring the resistance in voltage-clamp mode using the “Membrane Test” module of pClamp10 software (Molecular Devices, CA, USA). To prevent clogging of the tip, we applied positive pressure to the pipette via a 1.0ml syringe. After a seal was established with a cell, we ruptured its membrane by gently applying negative pressure to the pipette to establish a whole-cell configuration. We did not correct for leak or junction potentials. Current and voltage signals were amplified using a DC amplifier (MultiClamp 700) and digitized using Digidata 1440a system (Molecular Devices, CA, USA) at 10kHz and stored for subsequent off-line analysis.

Drug application

All drugs were purchased from Sigma Aldrich (USA) and stored as stock solution at -20^{o}C. The drugs were applied through a perfusion system equipped with TTL-driven pinch-valves (Automate Scientific, CA, USA). The TTL signal used to open the valve and start the drug infusion was delivered via the pClamp10 software and also fed as an analog input to the digitizer, which ensured synchronization of the injection with the recordings.

Optogenetic stimulation

In some experiments we used a TTL-driven white LED (Sutter Instrument, USA) to excite the neurons or terminals expressing the light sensitive protein Channelrhodopsin 2. The white light was filtered to generate a 460nm wave length. The light pulse was delivered through the X40 objective of the microscope with pulse duration of 5-20ms at 0.1Hz. The TTL signal that activated the light pulse was incorporated into the acquisition protocol and also fed as an analog input to the digitizer.

Surgical procedures

Sciatic nerve injury: To produce mechanical hypersensitivity we used the spared nerve injury (SNI) neuropathic pain model, as described previously (Shields et al., 2003). Briefly, mice were anesthetized with 2% isofluorane and then we made a small incision on the left thigh, which exposed the sciatic nerve proximal to its trifurcation. Using 8-0 silk suture (Ethicon, Summerville, NJ), a tight ligature was tied around the

common peroneal and sural nerve branches of the sciatic nerve, followed by their transection and removal of a 1.0 mm segment distal to the ligature. This procedure spares the tibial nerve. Overlying muscle and skin layers were close separately with 6-0 silk suture and suture clips (Harvard Apparatus, Holliston, MA), respectively.

Excitotoxic lesions: We define the "ACC" as that corresponding to Broadmann Area 24. To ablate the ACC, mice were deeply anesthetized with 2% isofluorane and placed securely in a stereotaxic instrument (Model 1900 Kopf). Scalp hair was removed with Nair, the scalp cleaned with betadine and ethanol, after which a midline skin incision was made. A burr hole was made for stereotaxic targeting of the rostral anterior cingulate cortex (rACC): -0.4mm, 1.30mm, -1.8mm; to target the posterior anterior cingulate cortex (pACC): -0.4mm, 0.75mm, -1.8mm. We then microinjected ibotenic acid (5.0mM, 500µL/side, 200nL/min) using a pulled 1.0mm OD glass micropipette. In control animals we made an incision of the scalp after which the skin was closed with staples. All animals were given 6 days to recover before any behavioral experiments were conducted.

Transplantation of medial ganglionic eminence cells: The methods used to transplant MGE cells have been described previously (Alvarez-Dolado et al., 2006). For transplantation, 6- to 8-week-old mice (naive or 1 week after paclitaxel treatment) were anesthetized under 2% isofluorane after which the head was secured in a Model 1900 Stereotaxic instrument (KOPF). A cell suspension containing $53x10^4$ MGE cells was loaded into a 1.0 mm OD glass micropipette (prefilled with mineral oil). The micropipette was connected to a Remote infuse/withdrawal Pump 11 Elite Nanomite Programmable

Syringe pump (Harvard Apparatus, Holliston, MA) mounted on a stereotactic apparatus. The following stereotactic coordinates were used for the microinjection (6 injections at of 60nL each, two sites on each side, at 3 different depths -1.8mm, -1.65mm, and -1.5mm) of the cell suspension were as follows: to target the rostral anterior cingulate cortex: -0.4mm, 1.30mm to 1.50mm, and -1.8mm to -1.5mm; to target the posterior anterior cingulate cortex: -0.4mm, 0.60mm to 0.75mm, and -1.8 to -1.5mm. Control groups were injected with an equivalent volume of Dulbecco's Modified Eagle's Medium (DMEM). The rate of injection was 100nL/min. The wound was closed with staples and the animals were allowed to recover from anesthesia under a heat lamp before they were returned to their home cages. Four weeks after the transplant, we measured the mechanical thresholds of each paw. As found previously, in uninjured animals, the cells had no effect on mechanical thresholds. Importantly, none of the transplanted animals exhibited signs of motor impairment.

Circuit tracing in the ACC: These experiments were directed at the circuits that engage transplanted cells. Here we transplanted naive mice with MGE cells that were genetically modified to express Cre recombinase and the TVA receptor, which can be targeted by pseudotyped rabies virus. The MGE cells were isolated from I12b-Cre:ROSA-tdTomato:$_{loxP}$STOP$_{loxP}$-TVA_RG mice. Thirty days after transplant, we microinjected 500nL of EnvA-Rabies-GFP_ΔG virus using the same coordinates. Two-weeks later, the mice were perfused and the brain tissue processed for immunohistochemistry. tdTOM+/GFP+ cells marked MGE cells that took up the rabies virus ("starter cells"); tdTOM+ marked MGE cells that did not take up virus; GFP+ only cells marked cells that are presynaptic to the MGE cells.

Quantitative PCR: Fourteen and 30 days after nerve injury (SNI) (n = 4 per group) a group of mice were killed by decapitation and the brain quickly dissected and frozen. Tissue from naive (uninjured) mice (n = 4) served as controls. We cut 300μM Vibratome sections that included cerebral cortex and collected 2.0 mm ID punches from the rACC (bilateral), just rostral to where the corpus collosum no longer crosses the midline. A comparable region was dissected from naïve animals. We used the RNeasy Minikit from Qiagen to extract mRNA, after which 200 ng of purified mRNA was reverse-transcribed into cDNA using Superscript II (Invitrogen, Carlsbad, CA, USA). mRNA levels for GAD65, GAD67, GAT3, $GABA_AR$- α1, $GABA_BR$_B1, VGAT and GAPDH were quantified with a Realplex^2 real-time PCR system (Eppendorf, Hamburg, Germany) using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Ratios of each gene to GAPDH mRNA were compared and analyzed by a one-way ANOVA followed by a Tukey's multiple comparison test. Asterisks (*) indicate statistically significant differences between groups, with * = $p < 0.05$ and **= $p < 0.01$.

Behavior

Hargreaves Test: Mice were habituated on a wire mesh for 30 min and then transferred to the Hargreaves apparatus for another 30 min before testing. Once mice were calm and completely still, we placed a probe that emits radiant heat beneath the plantar surface of the hindpaw. The time it took for the mouse to withdrawal the paw from the heat source was measured (latency). Each paw was tested 5 times with at least 2 minutes between each test. Laser power was set to 71 (arbitrary units, cut-off was 20sec).

Mechanical threshold: For all groups of animals tested, we recorded 3 days of baseline readings before any surgical procedure. Animals were habituated on a wire mesh for 2 hours, and then their mechanical withdrawal thresholds were tested using the up-down method with Von Frey filaments (1.65, 2.44, 2.84, 3.22, 3.61, 3.84, 4.08, 4.31g). Thresholds post SNI and again post transplant were normalized to each animal's Pre-SNI baseline threshold. If mechanical thresholds dropped by at least 50% from baseline, animals were considered to manifest mechanical allodynia.

Formalin Test: Mice were habituated on a wire mesh for 1 hour and then transferred to a clear Plexiglass surface for another 30min. After the habituation period, we injected 20μL 1.0% formalin into the plantar surface of the left hind paw and then the mice were immediately returned to the wire mesh. The mice were video taped (for 45 min) from beneath the mesh using a mirror that provided the experimenter with a clear view of behaviors. The total time spent displaying nocifensive behaviors (licking, guarding and shaking of the injured paw) was scored in 5 min bins, from 0 to 45 min post-injection.

Conditioned Place Paradigm: For the conditioned place preference paradigm (CPP), we used 3-chambered custom designed apparatus (Tap Plastics). Each box had different visual (dots vs. stripes), olfactory (lemon vs. vanilla extract), and flooring (smooth vs. rough) that distinguished the two end chambers. The middle chamber, which served as the neutral chamber, no visual, tactile or odor cues associated with it. During habituation sessions, and on Pretest or Test days, the mice were always placed first into the neutral chamber.

On **Day 1**, the mice were habituated with full access to all parts of the test apparatus in the afternoon (3:30PM – 4:00PM). The next morning (9:00AM-9:30AM), **Day 2**, the mice were habituated to the environment and in the afternoon their baseline preference for each chamber was recorded for 30 min ("Pretest") Importantly, in establishing the preference for each chamber, only the second 15 min of the video was scored . After Pretest readings were determined, a presumptive analgesic "pain reliever" (e.g. gabapentin) was paired with the less preferred chamber, and saline was paired with the more preferred chamber. On the conditioning days, **Day 3 and 4**, morning (9:00AM), the control substance (i.e. saline) was administered – the mice were injected i.p. with saline (volume equivalent to that required for the drug injection) and 45 min later were restricted for 30min to one of the two chambers. That afternoon (3:30PM) we performed conditioning sessions for the experimental drug (i.e. gabapentin) inhere the mice were restricted to the other chamber for 30 min, after the test drug was administered. We injected gabapentin either intraperitoneally 45 min before the mice were placed into the chamber or icv, immediately before the animal was placed into the chamber. On **Day 5**, the "Test" day, the mice were placed into the middle chamber and allowed to roam freely between all chambers of the apparatus and their preference for each chamber during the second 15 min of the trial was recorded.

To calculate the CPP Score, we subtracted the time (seconds) spent in each chamber of the apparatus on the Pretest day from that of the Test day (**CPP Score** = Test-Pretest). CPP Scores for each chamber (i.e. drug-paired side or vehicle-paired side) for

animals within each experimental group (i.e. lesioned vs. control) were pooled. Within each group (sham surgery vs. experimental surgery) CPP scores for the drug paired chamber vs. vehicle-paired chamber were analyzed with paired t-tests.

Figure 1: rACC do not alter the sensory-discriminative component of pain.

(a) Location of lesions shown in b.

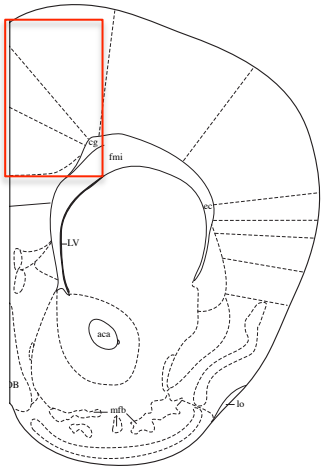
(b) Representative example of excitotoxic lesions. Loss of neurons, marked with antibodies to NeuN (green), and inflitration of microglia marked with antibodies to Iba! (red) define the extent of the lesion. (Note that lesions were bilateral).

(c) Left and right paw withdrawal latencies to radiant heat in animals with (n = 13) and without (n = 6) rACC ibotenic acid (IBO) lesions. There is no significant difference in acute thermal thresholds (One-way ANOVA, Dunnett's multiple comparison test, n.s.) in the two groups.

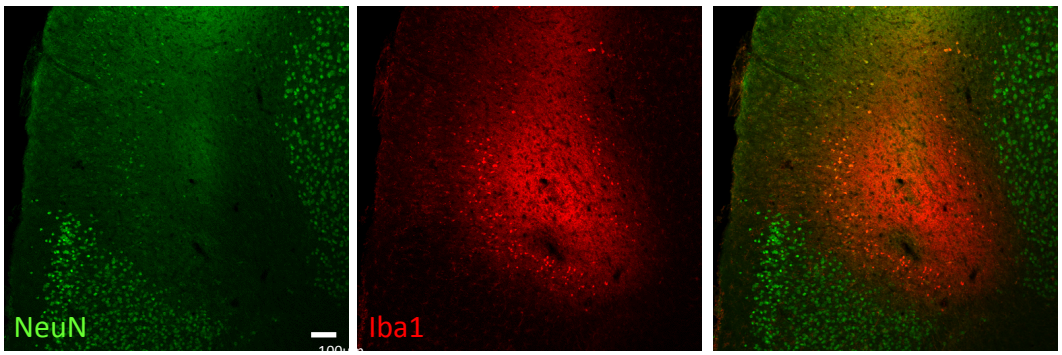
(d) Time spent licking the hindpaw after injection of formalin, in mice with (lesion, n = 12) and without (control, n = 9) rACC lesions. No significant difference was found between the groups (2way ANOVA, Sidak's multiple comparison test, n.s.) at any time point. Data are presented as the mean +/- s.e.m. of total licking time within each 5 minute bin.

(e,f) The rACC does not contribute to the maintenance of nerve-injury induced mechanical allodynia. Mechanical threshold data of the ipsilateral paw **(e)** and contralateral paw **(f)** are presented. Mice with (n = 7, red bars) and without (n=13, black bars) ACC lesions manifest mechanical allodynia equally after SNI. All mice were tested before injury (pre-SNI), after injury (post-SNI), and 1 week after rACC lesion (post-Lesion). There is no significant difference between groups (RM Two-way ANOVA, Sidak's multiple comparisons test). Data are represented as mean ± s.e.m.

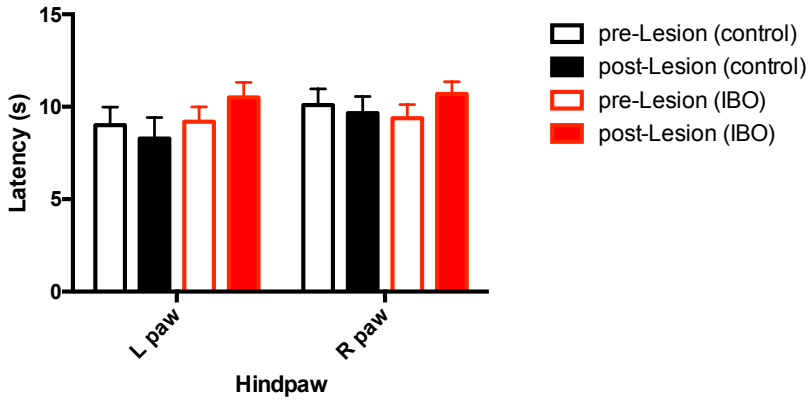
a.


cg
fmi
LV
aca
mfb
lo

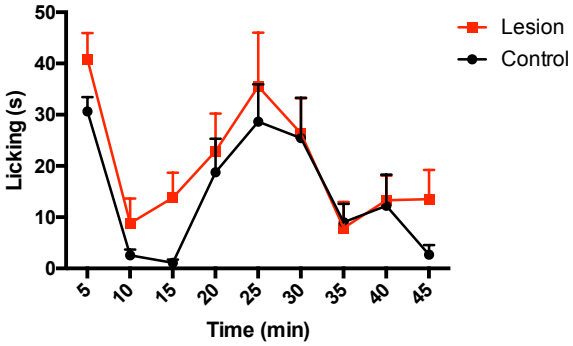
b.


NeuN
Iba1

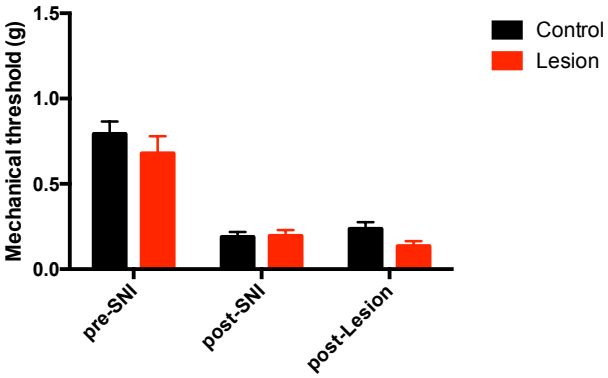
c.


15
10
5
0
Latency (s)
L paw
R paw
Hindpaw
pre-Lesion (control)
post-Lesion (control)
pre-Lesion (IBO)
post-Lesion (IBO)

d.


Lesion
Control
Licking (s)
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
Time (min)

e.


Control
Lesion
Mechanical threshold (g)
1.5
1.0
0.5
0.0
pre-SNI
post-SNI
post-Lesion

f.

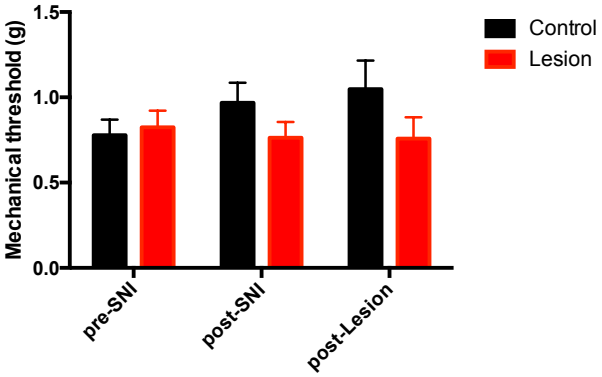
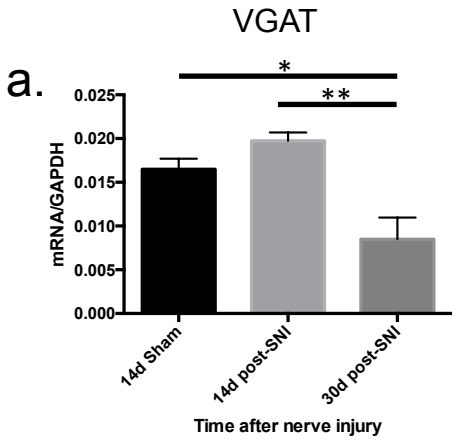

Control
Lesion
Mechanical threshold (g)
1.5
1.0
0.5
0.0
pre-SNI
post-SNI
post-Lesion

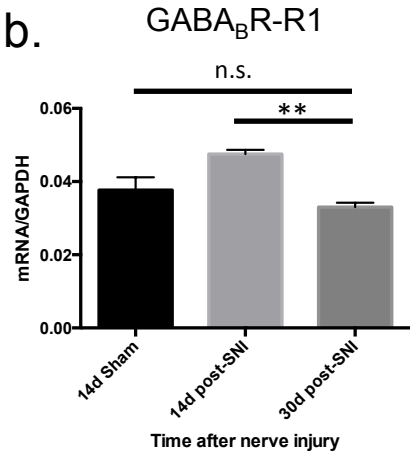
Figure 2: Peripheral nerve injury downregulates VGAT mRNA is in the rACC of mice

(a) q-PCR data showing the level of VGAT mRNA decreases in nerve-injured animals (n=4), compared to sham (n=4), 30 days after nerve-injury. All values were normalized to GAPDH levels (One-way ANOVA, Tukey's multiple comparisons test, $p < 0.05$).

(b) $GABA_B$-R1 mRNA is transiently upregulated in the rACC 14 days after nerve-injury, but returns to baseline level by 30 days post-injury (One-way ANOVA, Tukey's multiple comparisons test).

(c) Histogram illustrates lack of mRNA change rACC for GAD65, GAD67, GAT3, or $GABA_A$-α1 mRNA levels after nerve injury (One-way ANOVA, Tukey's multiple comparisons test). Data are represented as mean ± s.e.m.


VGAT
a.
*
**
mRNA/GAPDH
0.025
0.020
0.015
0.010
0.005
0.000
14d Sham
14d post-SNI
30d post-SNI
Time after nerve injury


b.
GABA$_B$R-R1
n.s.
**
mRNA/GAPDH
0.06
0.04
0.02
0.00
14d Sham
14d post-SNI
30d post-SNI
Time after nerve injury

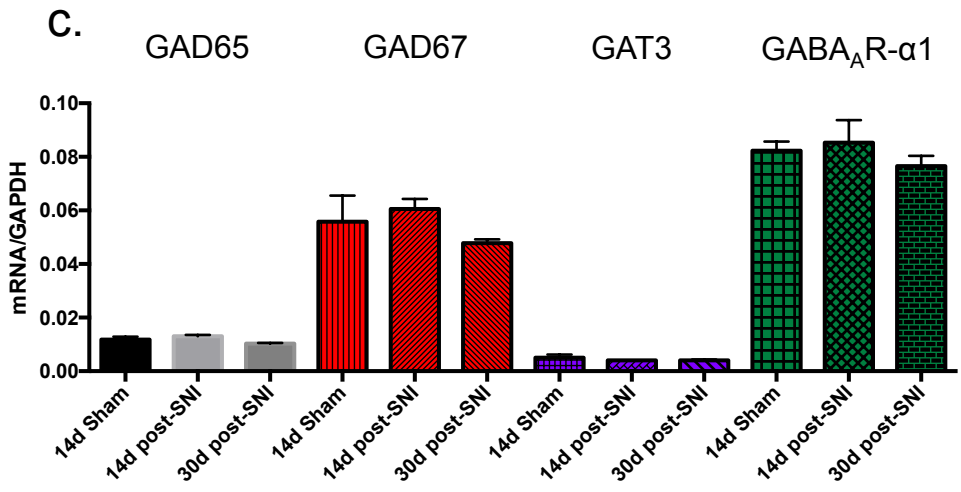

c.
GAD65
GAD67
GAT3
GABA$_A$R-α1
mRNA/GAPDH
0.10
0.08
0.06
0.04
0.02
0.00
14d Sham
14d post-SNI
30d post-SNI
14d Sham
14d post-SNI
30d post-SNI
14d Sham
14d post-SNI
30d post-SNI
14d Sham
14d post-SNI
30d post-SNI

Figure 3. MGE-cell transplants in the ACC of paclitaxel treated mice.

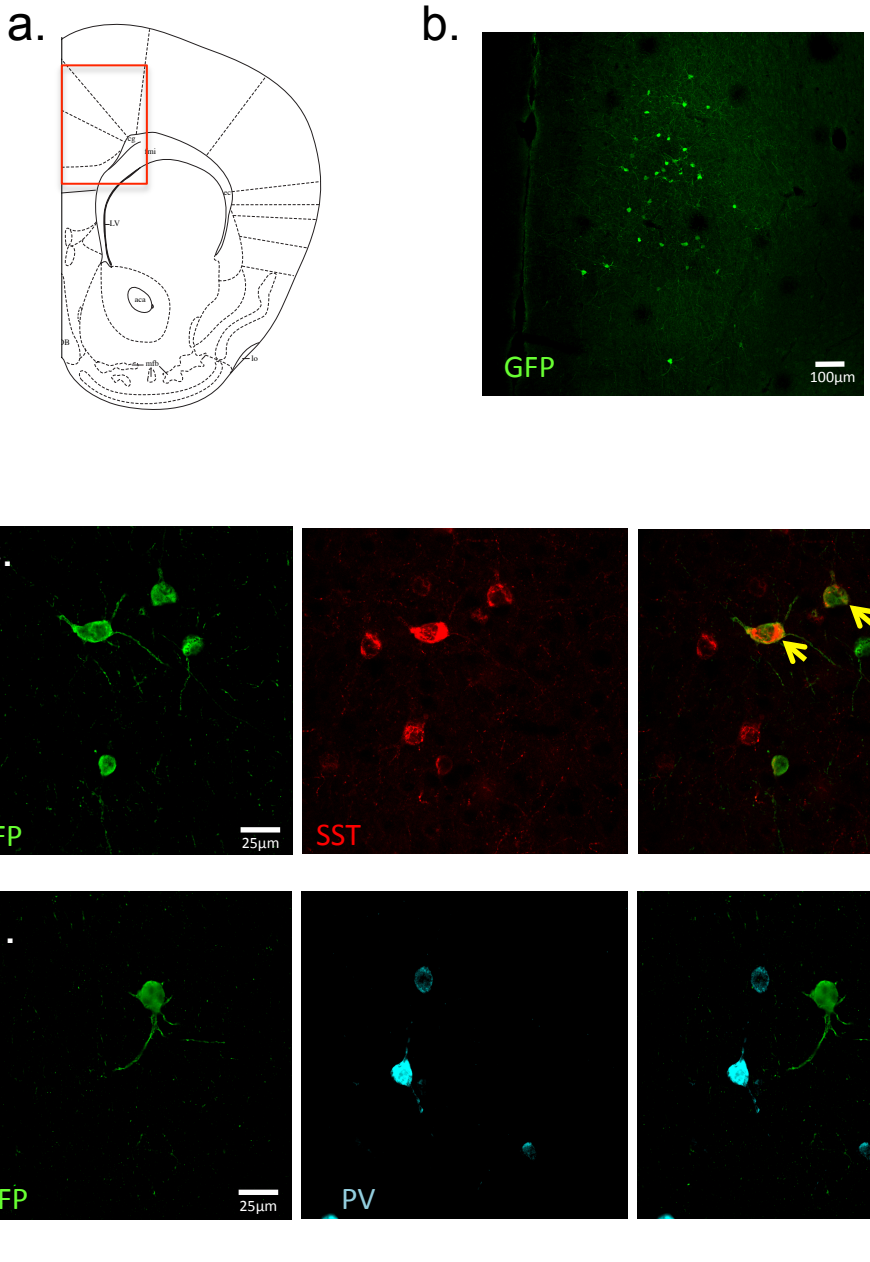
MGE-derived cells constitutively express GFP (green).

(a) Diagram depicting location of MGE transplants illustrated in b.

(b) 30 days after transplantation, there is an aggregation of GFP+ MGE cells at or near the injection site. Many cells migrated up to 1800μm from the injection site, showing a strong preference for the rostrocaudal direction. The transplants were injected bilaterally. The majority of GFP+ MGE cells spread throughout all cortical layers. Scale bar equals 100 μm.

(c) Transplanted cells assume a GABAergic phenotype in the paclitaxel-treated adult mice. MGE-derived cells (green) expressed somatostatin (red), but not **(d)** parvalbumin (blue), both markers of cortical GABAergic interneurons. Arrows point to double-labeled cells. Scale bar equals 25μm.

(e) Histogram illustrating percentage of GFP+ cells that were immunoreactive for SST, PV, GABA, and NeuN. Cells were counted in 3 mice.


a.
b.
GFP
100µm
c.
GFP
25µm
SST
d.
GFP
25µm
PV

e.

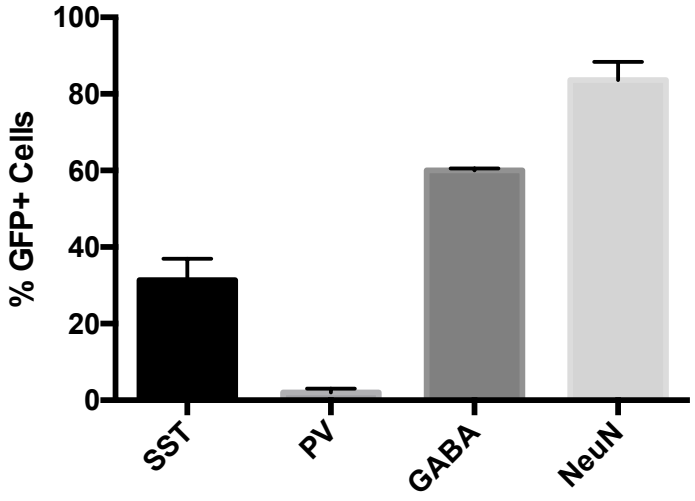


Figure 4: MGE-derived transplants have properties characteristic of different subsets of GABAergic inhibitory interneurons and are synaptic related to pyramidal cells

(a,b) Current-clamp recordings from GFP+ MGE neurons showing three different spiking patterns (FS: n =8/18, Non-FS: n=10/18). The inset in (b, right) shows the difference in action potential morphology between the fast (black) and non-fast spiking (green) neurons.

(c) The schematic shows the paradigm that was used to establish functional connectivity between the MGE (black) and host (red) neurons. The top right panel shows the action potentials discharge evoked in the MGE neuron (top) and the resulting IPSC in the host pyramidal neuron (bottom, black), which could be blocked by gabazine (blue) (n = 3/17 of FS: 0/9 non-FS).

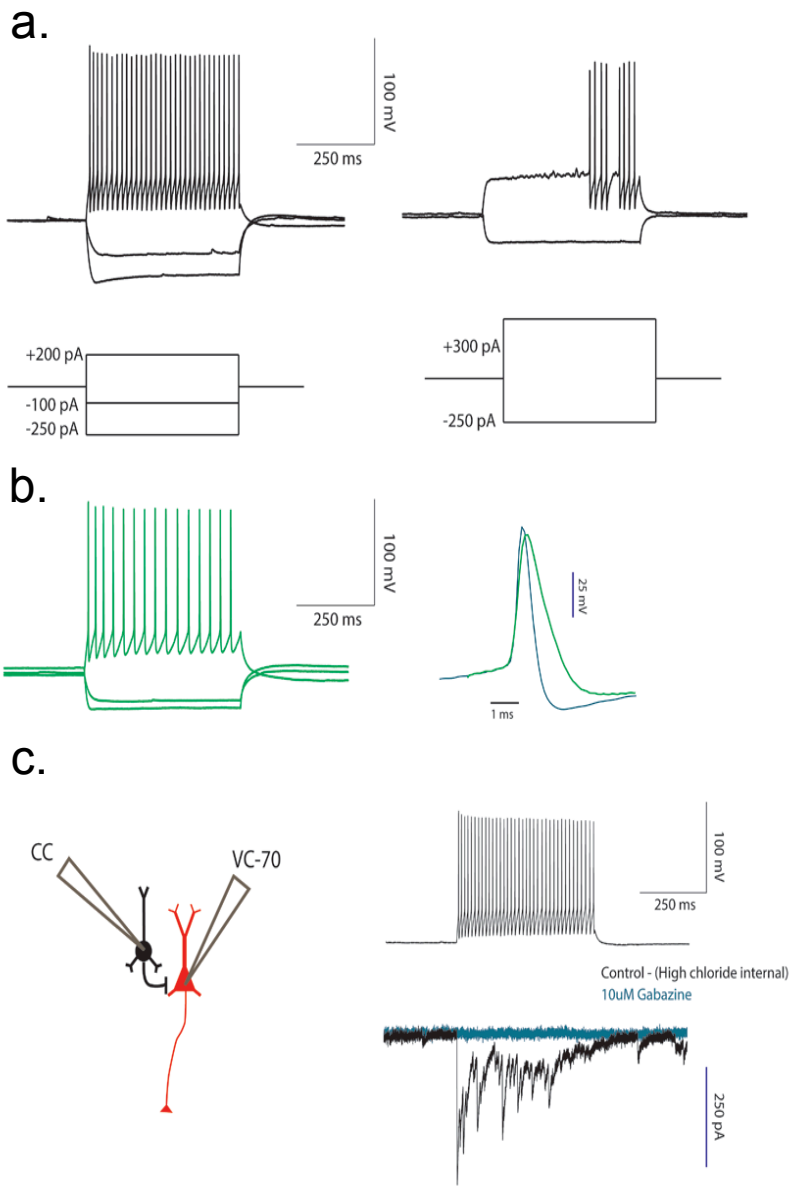

a.
100 mV
250 ms
+200 pA
-100 pA
-250 pA
+300 pA
-250 pA
b.
100 mV
250 ms
25 mV
1 ms
c.
CC
VC-70
100 mV
250 ms
Control - (High chloride internal)
10uM Gabazine
250 pA

Figure 5: MGE-evoked inhibition is GABA-A receptor mediated

(a) Schematic shows the paradigm used to facilitate identification and pharmacological analysis of MGE-host synaptic connections (see Methods).

(b) Examples of optogenetically-evoked IPSCs in host pyramidal neurons at baseline, after application of bicuculline and 10min wash.

(c) The magnitude of the evoked IPSCs (expressed as area under curve) was significantly reduced by bicuculline and recovered to baseline after wash.

(d, e): The $GABA_BR$ antagonist CGP55845 did not alter the magnitude of the optogenetically-evoked IPSCs.

(f) A blue light pulse (100ms), time-dependently blocked evoked firing in the host neuron.

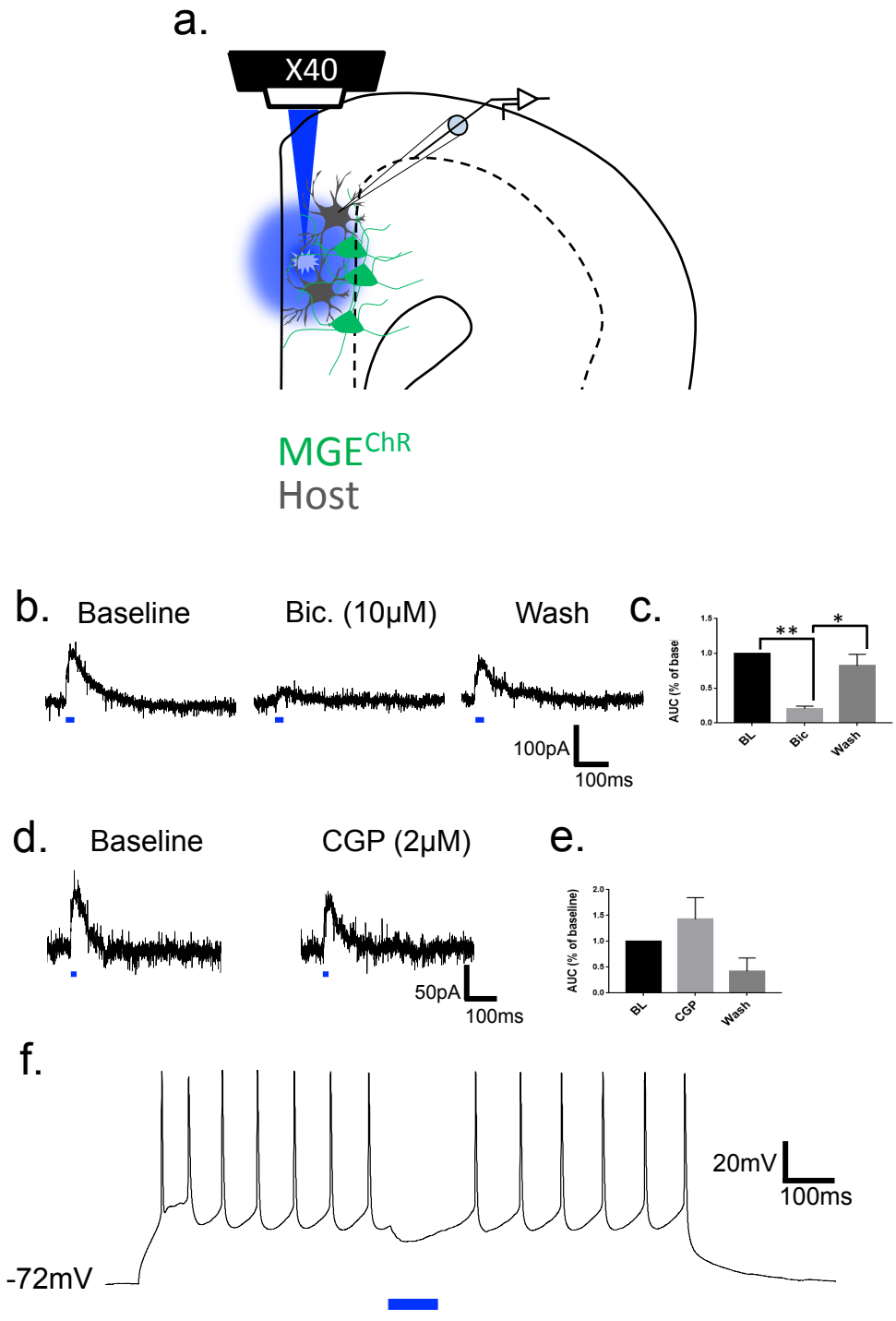
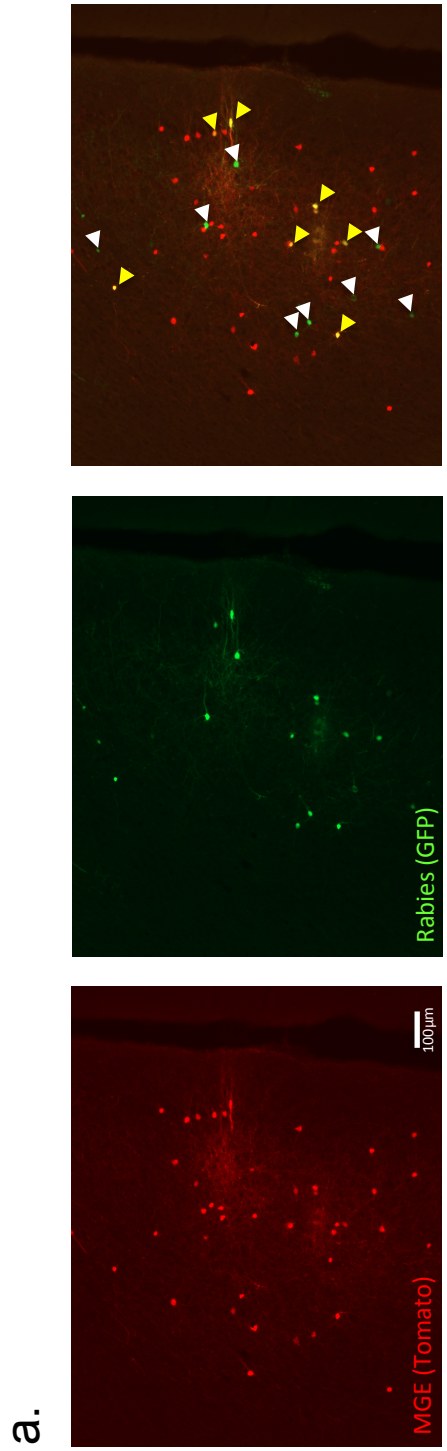

a.
X40
MGE^ChR
Host
b.
Baseline
Bic. (10μM)
Wash
100pA
100ms
c.
AUC (% of base
BL
Bic
Wash
d.
Baseline
CGP (2μM)
50pA
100ms
e.
AUC (% of baseline)
BL
CGP
Wash
f.
20mV
100ms
-72mV

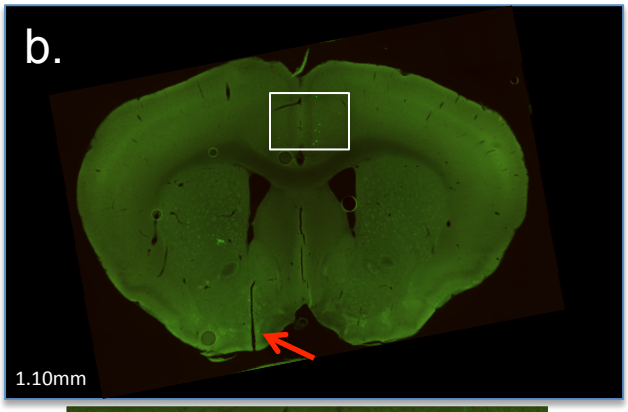
Figure 6: MGE-derived transplants integrate into host rACC circuitry and receive afferent connections from distant brain regions.

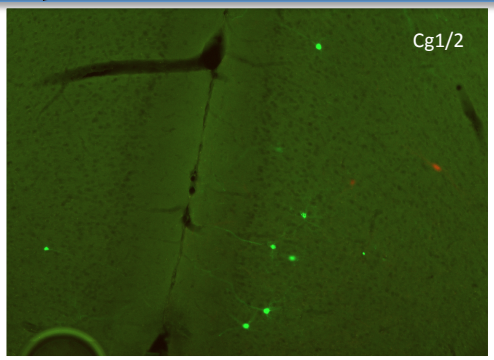
(a) Immunofluorescence showing TVA-R expressing MGE cells (tomato +) in the rACC 30 days after transplant. Because the Rabies-GFP-ΔG virus (green) is pseudotyped to express EnVa, it can only enter TVA-R expressing cells (yellow starter cells, yellow arrowheads) and only move one synapse retrogradely from the starter cell. Therefore, presynaptic cells to the MGE cell are labeled with GFP only (green, white arrowheads). MGE cells were transplanted unilaterally.

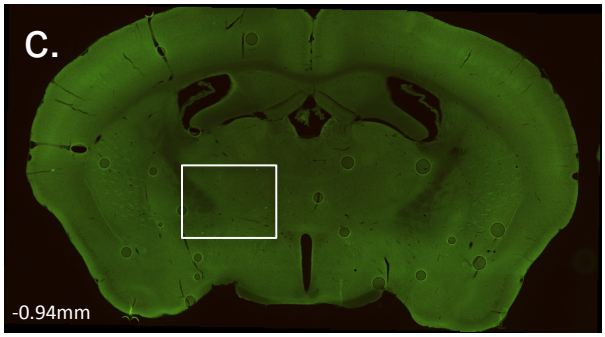
(b) Image illustrates neurons that project to the MGE cells located in the pACC. Note that GFP+ cells are located bilaterally, which demonstrates that MGE transplants receive inputs from ipsilateral as well as contralateral cortex (see inset).

(c) A caudal brain section showing thalamic (1 & 2), hypothalamic (lateral hypothalamus, LH), and amygdala (AMYG) GFP+ labeled neurons that project to rACC MGE cells. Brain regions where GFP+ cells were found were comparable across mice (n=4). A cut made in the tissue (red arrow) marks the side contralateral to the transplant. The rostrocaudal stereotaxic coordinates of the section is located in the lower left corner.


a.
MGE (Tomato)
100µm
Rabies (GFP)


b.
1.10mm


Cg1/2


c.
-0.94mm

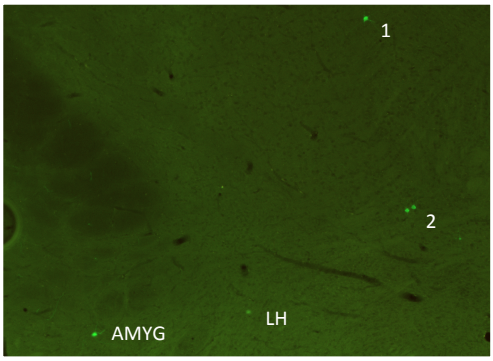
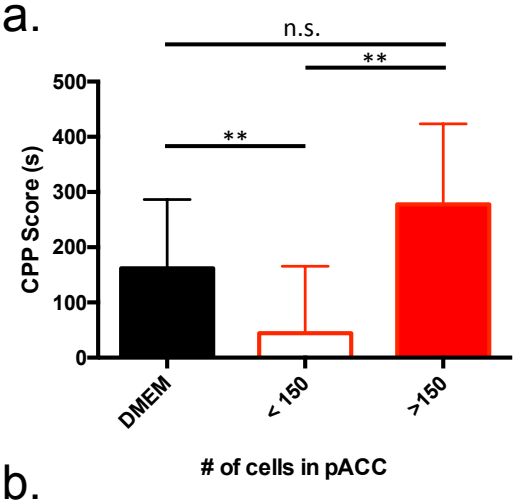

1
2
AMYG
LH

Figure 7: MGE-derived transplants in the rACC are pain relieving and this effect is reversed when MGE cells concurrent populate the pACC.

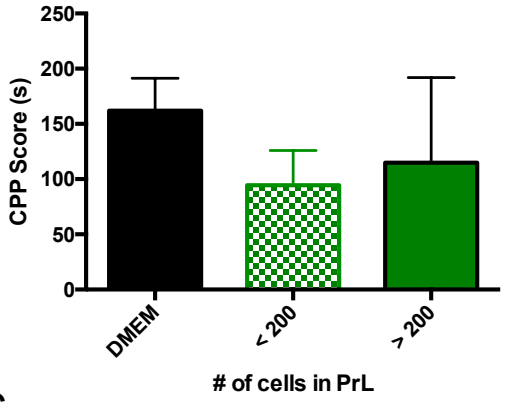
(a) Histogram illustrates the CPP Score (s) of DMEM (control; n = 18) and MGE-transplanted animals (n = 31). Mice with less than 150 MGE cells in the pACC (n=24) show a significant loss of preference for gabapentin ($p < 0.01$), indicating that MGE cells in the rACC have a strong pain-relieving (anti-aversive) effect. Conversely, mice with more than 150 cells in the pACC (n = 7), lose the analgesic benefit of MGE-mediated inhibition in the rACC (One-way ANOVA, Kruskal-Wallis test with Dunn's multiple comparisons correction). There were no transplanted mice in which cells only populated pACC.

(b) CPP sores of animals with DMEM (n=18), < 200 (n=26) or > 200 (n=5) GFP+ cells in the PrL and **(c)** M2 (<200 n =18, >200 n = 13). There was no effect of MGE cells located in these two regions on CPP Score, indicating that MGE-mediated inhibition had no analgesic or proalgesic role in these regions (One-way ANOVA, Kruskal-Wallis test with Dunn's multiple comparisons correction, n.s.). Data are represented as mean ± s.e.m.

a.


n.s.
**
**
CPP Score (s)
500
400
300
200
100
0
DMEM
< 150
>150
# of cells in pACC

b.


CPP Score (s)
250
200
150
100
50
0
DMEM
< 200
> 200
# of cells in PrL

c.

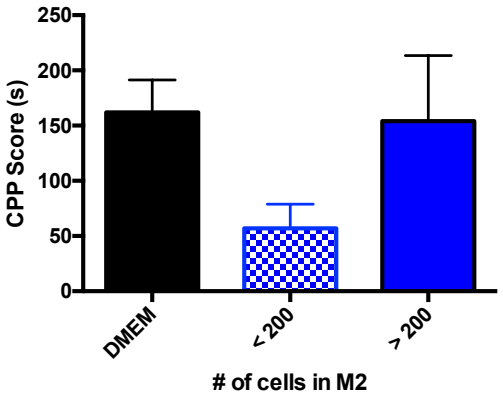
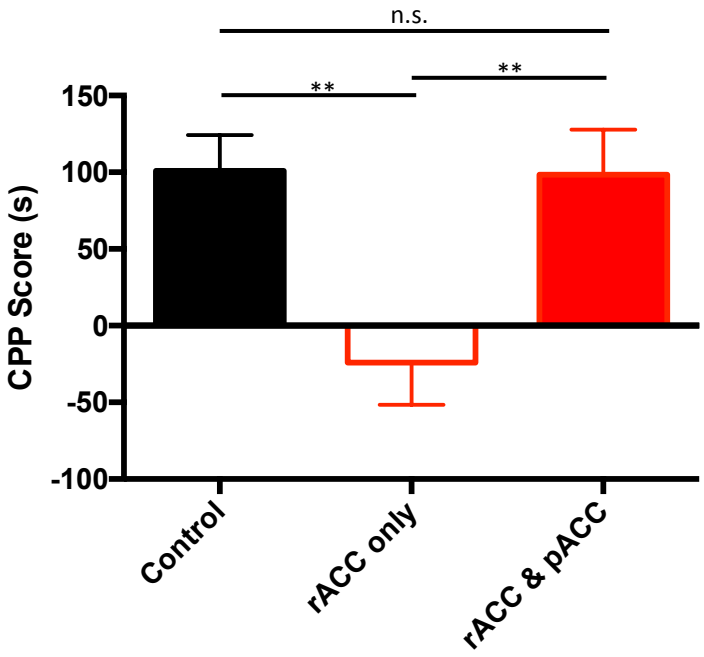

CPP Score (s)
250
200
150
100
50
0
DMEM
< 200
> 200
# of cells in M2

Figure 8: Combined lesions of the rACC and pACC reverse the analgesic effect of rACC lesions. Histogram illustrates the CPP score of mice with lesions limited to the rACC (n = 5), to the rACC and pACC lesions (n = 12), or control (n = 18). Animals with restricted rACC lesions lose their preference for gabapentin, compared to the control animals ($p < 0.01$) and to the rACC/pACC lesion animals ($p < 0.01$). This indicates that loss of preference for gabapentin only occurs with lesions limited to the rACC. In contrast, animals with combined rACC and pACC lesions have a CPP score for gabapentin that did not differ from that of control animals. This indicates that there remains a preference for gabapentin, i.e. the mice experience tonic pain aversiveness (One-way ANOVA, Kruskal-Wallis test with Dunn's multiple comparisons correction). Data are represented as mean ± s.e.m.


n.s.
**
**
150
100
50
0
-50
-100
CPP Score (s)
Control
rACC only
rACC & pACC

Conclusion

The experiments described in this thesis examined two different mechanisms of supraspinally-mediated pain relief. In Chapter 1, we asked whether supraspinal gabapentin induce pain relief, and the extent to which the pain relief was independent of the initiation of noradrenergic descending controls. First, we found that gabapentin is indeed pain relieving in mice with neuropathic pain, as it induced a place preference in injured, but not uninjured, mice. Second, we found that blocking descending noradrenergic controls, by administering yohimbine at the level of the spinal cord, significantly reduced the pain relieving effect of supraspinal gabapentin. We conclude, therefore, that supraspinal gabapentin induces pain relief by initiating descending, antinociceptive noradrenergic inhibitory controls, and that these controls are, in fact, necessary for the supraspinally-mediated analgesic effect of gabapentin.

How gabapentin engages the descending noradrenergic inhibitory controls is not clear. However, a review of the literature provides some insight. First, previous studies demonstrated that gabapentin is only effective in relieving pain following its binding in regions of the nervous system where there is a nerve injury-induced upregulation of its molecular target, α2δ-1. Second, and most importantly, electrophysiological studies demonstrated that nerve injury induces α2δ-1 upregulation in GABAergic neurons of the locus coeruleus. This upregulation likely results in an increased GABAergic inhibition of the noradrenergic descending controls, a process that would increase nociceptive transmission at the level of the spinal cord. As gabapentin binds to the α2δ-1 site, there

would be decreased GABAergic inhibitory control of LC neurons and a resultant increase in noradrenergic descending inhibitory control. The behavioral consequence of this supraspinal action of gabapentin on LC neurons would be manifest as an antinociceptive-initiated pain relief.

Although there is clearly widespread expression of the α2δ-1 subunit of Ca^{2+} channels, to date there are no published studies demonstrating a direct action of gabapentin in the ACC. We hypothesize, therefore, that despite the nerve-injury induced reduction of inhibitory GABAergic controls in the ACC, this is not paralleled by an upregulation of the α2δ-1 subunit. Given the apparent association of gabapentin's action with α2δ-1 upregulation, it follows that gabapentin would not be effective against hyperexcitable ACC neurons. This scenario could also explain why we did not see a gabapentin-mediated supraspinal pain relief that is independent of descending inhibitory controls.

In Chapter 2, we transplanted GABAergic progenitor cells of the MGE into the rostral ACC and demonstrated a significant pain relief in a neuropathic pain model. Using the CPP paradigm, we demonstrated that the nerve-injured mice no longer showed a preference for gabapentin. We conclude that reestablishing GABAergic inhibitory controls in the ACC can be remarkably effective against what we presume to be the affective component of the pain experience. Unexpectedly, we found that the pain relief was lost if some of the MGE cells that were targeted to the rACC migrated into the adjacent posterior ACC. This finding indicates that concurrent MGE-mediated inhibition of the rACC and pACC results in a state of tonic aversiveness, which manifests as a

place preference for the gabapentin. Based on these observations we proposed a model in which the pACC is not only the source of inhibitory feedback that regulates rACC neurons, but also onto an as yet unidentified brain region (or regions) that must also contribute to pain aversiveness. To our knowledge, this is the first demonstration that combined lesions of the rACC and pACC are pronociceptive. Future studies of the supraspinal mechanisms that contribute to pain relief should focus on the interactions between pACC and rACC neurons as well as between pACC neurons and other brain regions implicated in pain aversiveness. Taken together, these studies confirm the importance of regulating the rACC to achieve pain relief. But perhaps more importantly, these studies highlight the remarkable ability of MGE inhibitory progenitor cell transplants to provide long-term pain relief, initially in a preclinical model of neuropathic pain, but potentially in the clinical setting.

REFERENCES

Alvarez-Dolado, M., Calcagnotto, M.E., Karkar, K.M., Southwell, D.G., Jones-Davis, D.M., Estrada, R.C., Rubenstein, J.L., Alvarez-Buylla, A., Baraban, S.C., 2006. Cortical inhibition modified by embryonic neural precursors grafted into the postnatal brain. J Neurosci 26, 7380-7389.

Andrews, N., Loomis, S., Blake, R., Ferrigan, L., Singh, L., McKnight, A.T., 2001. Effect of gabapentin-like compounds on development and maintenance of morphine-induced conditioned place preference. Psychopharmacology (Berl) 157, 381-387.

Arntz, A., Claassens, L., 2004. The meaning of pain influences its experienced intensity. Pain 109, 20-25.

Backonja, M., Beydoun, A., Edwards, K.R., Schwartz, S.L., Fonseca, V., Hes, M., LaMoreaux, L., Garofalo, E., 1998. Gabapentin for the symptomatic treatment of painful neuropathy in patients with diabetes mellitus: a randomized controlled trial. JAMA 280, 1831-1836.

Baliki, M.N., Baria, A.T., Apkarian, A.V., 2011. The cortical rhythms of chronic back pain. J Neurosci 31, 13981-13990.

Baliki, M.N., Chialvo, D.R., Geha, P.Y., Levy, R.M., Harden, R.N., Parrish, T.B., Apkarian, A.V., 2006. Chronic pain and the emotional brain: specific brain activity associated with spontaneous fluctuations of intensity of chronic back pain. J Neurosci 26, 12165-12173.

Baliki, M.N., Mansour, A.R., Baria, A.T., Apkarian, A.V., 2014. Functional reorganization of the default mode network across chronic pain conditions. PLoS One 9, e106133.

Baraban, S.C., Southwell, D.G., Estrada, R.C., Jones, D.L., Sebe, J.Y., Alfaro-Cervello, C., Garcia-Verdugo, J.M., Rubenstein, J.L., Alvarez-Buylla, A., 2009. Reduction of seizures by transplantation of cortical GABAergic interneuron precursors into Kv1.1 mutant mice. Proc Natl Acad Sci U S A 106, 15472-15477.

Bernad, P.G., Ballantine, H.T., 1987. Computed tomographic analysis of bilateral cingulotomy for intractable mood disturbance and chronic pain. Comput Radiol 11, 117-123.

Biemond, A., 1956. The conduction of pain above the level of the thalamus opticus. AMA Arch Neurol Psychiatry 75, 231-244.

Braz, J.M., Juarez-Salinas, D., Ross, S.E., Basbaum, A.I., 2014. Transplant restoration of spinal cord inhibitory controls ameliorates neuropathic itch. J Clin Invest 124, 3612-3616.

Braz, J.M., Sharif-Naeini, R., Vogt, D., Kriegstein, A., Alvarez-Buylla, A., Rubenstein, J.L., Basbaum, A.I., 2012. Forebrain GABAergic neuron precursors integrate into adult spinal cord and reduce injury-induced neuropathic pain. Neuron 74, 663-675.

Braz, J.M., Wang, X., Guan, Z., Rubenstein, J.L., Basbaum, A.I., 2015. Transplant-mediated enhancement of spinal cord GABAergic inhibition reverses paclitaxel-induced mechanical and heat hypersensitivity. Pain 156, 1084-1091.

Calcagnotto, M.E., Ruiz, L.P., Blanco, M.M., Santos-Junior, J.G., Valente, M.F., Patti, C., Frussa-Filho, R., Santiago, M.F., Zipancic, I., Alvarez-Dolado, M., Mello, L.E., Longo, B.M., 2010a. Effect of neuronal precursor cells derived from medial ganglionic eminence in an acute epileptic seizure model. Epilepsia 51 Suppl 3, 71-75.

Calcagnotto, M.E., Zipancic, I., Piquer-Gil, M., Mello, L.E., Alvarez-Dolado, M., 2010b. Grafting of GABAergic precursors rescues deficits in hippocampal inhibition. Epilepsia 51 Suppl 3, 66-70.

Callaway, E.M., Luo, L., 2015. Monosynaptic Circuit Tracing with Glycoprotein-Deleted Rabies Viruses. J Neurosci 35, 8979-8985.

Chen, S.R., Pan, H.L., 2005. Effect of systemic and intrathecal gabapentin on allodynia in a new rat model of postherpetic neuralgia. Brain Res 1042, 108-113.

Christensen, D., Gautron, M., Guilbaud, G., Kayser, V., 2001. Effect of gabapentin and lamotrigine on mechanical allodynia-like behaviour in a rat model of trigeminal neuropathic pain. Pain 93, 147-153.

Coderre, T.J., Kumar, N., Lefebvre, C.D., Yu, J.S., 2005. Evidence that gabapentin reduces neuropathic pain by inhibiting the spinal release of glutamate. J Neurochem 94, 1131-1139.

Coffeen, U., Manuel Ortega-Legaspi, J., Lopez-Munoz, F.J., Simon-Arceo, K., Jaimes, O., Pellicer, F., 2011. Insular cortex lesion diminishes neuropathic and inflammatory pain-like behaviours. Eur J Pain 15, 132-138.

Cole, R.L., Lechner, S.M., Williams, M.E., Prodanovich, P., Bleicher, L., Varney, M.A., Gu, G., 2005. Differential distribution of voltage-gated calcium channel alpha-2 delta (alpha2delta) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. J Comp Neurol 491, 246-269.

Craig, A.D., Jr., Burton, H., 1981. Spinal and medullary lamina I projection to nucleus submedius in medial thalamus: a possible pain center. J Neurophysiol 45, 443-466.

Dado, R.J., Giesler, G.J., Jr., 1990. Afferent input to nucleus submedius in rats: retrograde labeling of neurons in the spinal cord and caudal medulla. J Neurosci 10, 2672-2686.

Dafny, N., Dong, W.Q., Prieto-Gomez, C., Reyes-Vazquez, C., Stanford, J., Qiao, J.T., 1996. Lateral hypothalamus: site involved in pain modulation. Neuroscience 70, 449-460.

Derbyshire, S.W., 2000. Exploring the pain "neuromatrix". Curr Rev Pain 4, 467-477.

Donahue, R.R., LaGraize, S.C., Fuchs, P.N., 2001. Electrolytic lesion of the anterior cingulate cortex decreases inflammatory, but not neuropathic nociceptive behavior in rats. Brain Res 897, 131-138.

Dong, W.K., Ryu, H., Wagman, I.H., 1978. Nociceptive responses of neurons in medial thalamus and their relationship to spinothalamic pathways. J Neurophysiol 41, 1592-1613.

Farmer, M.A., Baliki, M.N., Apkarian, A.V., 2012. A dynamic network perspective of chronic pain. Neurosci Lett 520, 197-203.

Field, M.J., Cox, P.J., Stott, E., Melrose, H., Offord, J., Su, T.Z., Bramwell, S., Corradini, L., England, S., Winks, J., Kinloch, R.A., Hendrich, J., Dolphin, A.C., Webb, T., Williams, D., 2006. Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc Natl Acad Sci U S A 103, 17537-17542.

Field, M.J., Holloman, E.F., McCleary, S., Hughes, J., Singh, L., 1997. Evaluation of gabapentin and S-(+)-3-isobutylgaba in a rat model of postoperative pain. J Pharmacol Exp Ther 282, 1242-1246.

Field, M.J., McCleary, S., Hughes, J., Singh, L., 1999. Gabapentin and pregabalin, but not morphine and amitriptyline, block both static and dynamic components of mechanical allodynia induced by streptozocin in the rat. Pain 80, 391-398.

Fisk, G.D., Wyss, J.M., 1999. Associational projections of the anterior midline cortex in the rat: intracingulate and retrosplenial connections. Brain Res 825, 1-13.

Foltz, E.L., White, L.E., Jr., 1962. Pain "relief" by frontal cingulumotomy. J Neurosurg 19, 89-100.

Gauchan, P., Andoh, T., Ikeda, K., Fujita, M., Sasaki, A., Kato, A., Kuraishi, Y., 2009. Mechanical allodynia induced by paclitaxel, oxaliplatin and vincristine: different effectiveness of gabapentin and different expression of voltage-dependent calcium channel alpha(2)delta-1 subunit. Biol Pharm Bull 32, 732-734.

Gaykema, R.P., Luiten, P.G., Nyakas, C., Traber, J., 1990. Cortical projection patterns of the medial septum-diagonal band complex. J Comp Neurol 293, 103-124.

Gee, N.S., Brown, J.P., Dissanayake, V.U., Offord, J., Thurlow, R., Woodruff, G.N., 1996. The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha2delta subunit of a calcium channel. J Biol Chem 271, 5768-5776.

Han, J.S., Neugebauer, V., 2004. Synaptic plasticity in the amygdala in a visceral pain model in rats. Neurosci Lett 361, 254-257.

Han, J.S., Neugebauer, V., 2005. mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. Pain 113, 211-222.

Hashmi, J.A., Baliki, M.N., Huang, L., Baria, A.T., Torbey, S., Hermann, K.M., Schnitzer, T.J., Apkarian, A.V., 2013. Shape shifting pain: chronification of back pain shifts brain representation from nociceptive to emotional circuits. Brain 136, 2751-2768.

Hayashida, K., DeGoes, S., Curry, R., Eisenach, J.C., 2007. Gabapentin activates spinal noradrenergic activity in rats and humans and reduces hypersensitivity after surgery. Anesthesiology 106, 557-562.

Helmstetter, F.J., 1992. The amygdala is essential for the expression of conditional hypoalgesia. Behav Neurosci 106, 518-528.

Helmstetter, F.J., Bellgowan, P.S., 1993. Lesions of the amygdala block conditional hypoalgesia on the tail flick test. Brain Res 612, 253-257.

Hendrich, J., Van Minh, A.T., Heblich, F., Nieto-Rostro, M., Watschinger, K., Striessnig, J., Wratten, J., Davies, A., Dolphin, A.C., 2008. Pharmacological disruption of calcium channel trafficking by the alpha2delta ligand gabapentin. Proc Natl Acad Sci U S A 105, 3628-3633.

Hiller, J.M., Fan, L.Q., 1996. Laminar distribution of the multiple opioid receptors in the human cerebral cortex. Neurochem Res 21, 1333-1345.

Horikawa, K., Kinjo, N., Stanley, L.C., Powell, E.W., 1988. Topographic organization and collateralization of the projections of the anterior and laterodorsal thalamic nuclei to cingulate areas 24 and 29 in the rat. Neurosci Res 6, 31-44.

Hsieh, J.C., Stone-Elander, S., Ingvar, M., 1999. Anticipatory coping of pain expressed in the human anterior cingulate cortex: a positron emission tomography study. Neurosci Lett 262, 61-64.

Hunter, J.C., Gogas, K.R., Hedley, L.R., Jacobson, L.O., Kassotakis, L., Thompson, J., Fontana, D.J., 1997. The effect of novel anti-epileptic drugs in rat experimental models of acute and chronic pain. Eur J Pharmacol 324, 153-160.

Hutchison, W.D., Davis, K.D., Lozano, A.M., Tasker, R.R., Dostrovsky, J.O., 1999. Pain-related neurons in the human cingulate cortex. Nat Neurosci 2, 403-405.

Ikeda, R., Takahashi, Y., Inoue, K., Kato, F., 2007. NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain 127, 161-172.

Jasmin, L., Rabkin, S.D., Granato, A., Boudah, A., Ohara, P.T., 2003. Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. Nature 424, 316-320.

Johansen, J.P., Fields, H.L., 2004. Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. Nat Neurosci 7, 398-403.

Johansen, J.P., Fields, H.L., Manning, B.H., 2001. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. Proc Natl Acad Sci U S A 98, 8077-8082.

Jones, B.F., Groenewegen, H.J., Witter, M.P., 2005. Intrinsic connections of the cingulate cortex in the rat suggest the existence of multiple functionally segregated networks. Neuroscience 133, 193-207.

Kawaguchi, Y., Kondo, S., 2002. Parvalbumin, somatostatin and cholecystokinin as chemical markers for specific GABAergic interneuron types in the rat frontal cortex. J Neurocytol 31, 277-287.

Koyama, T., Tanaka, Y.Z., Mikami, A., 1998. Nociceptive neurons in the macaque anterior cingulate activate during anticipation of pain. Neuroreport 9, 2663-2667.

LaGraize, S.C., Borzan, J., Peng, Y.B., Fuchs, P.N., 2006. Selective regulation of pain affect following activation of the opioid anterior cingulate cortex system. Exp Neurol 197, 22-30.

LaGraize, S.C., Fuchs, P.N., 2007. GABAA but not GABAB receptors in the rostral anterior cingulate cortex selectively modulate pain-induced escape/avoidance behavior. Exp Neurol 204, 182-194.

Laughlin, T.M., Tram, K.V., Wilcox, G.L., Birnbaum, A.K., 2002. Comparison of antiepileptic drugs tiagabine, lamotrigine, and gabapentin in mouse models of acute, prolonged, and chronic nociception. J Pharmacol Exp Ther 302, 1168-1175.

Li, C.Y., Zhang, X.L., Matthews, E.A., Li, K.W., Kurwa, A., Boroujerdi, A., Gross, J., Gold, M.S., Dickenson, A.H., Feng, G., Luo, Z.D., 2006. Calcium channel alpha2delta1 subunit mediates spinal hyperexcitability in pain modulation. Pain 125, 20-34.

Li, X.Y., Ko, H.G., Chen, T., Descalzi, G., Koga, K., Wang, H., Kim, S.S., Shang, Y., Kwak, C., Park, S.W., Shim, J., Lee, K., Collingridge, G.L., Kaang, B.K., Zhuo, M., 2010. Alleviating neuropathic pain hypersensitivity by inhibiting PKMzeta in the anterior cingulate cortex. Science 330, 1400-1404.

Luo, Z.D., Calcutt, N.A., Higuera, E.S., Valder, C.R., Song, Y.H., Svensson, C.I., Myers, R.R., 2002. Injury type-specific calcium channel alpha 2 delta-1 subunit up-regulation in rat neuropathic pain models correlates with antiallodynic effects of gabapentin. J Pharmacol Exp Ther 303, 1199-1205.

Luo, Z.D., Chaplan, S.R., Higuera, E.S., Sorkin, L.S., Stauderman, K.A., Williams, M.E., Yaksh, T.L., 2001. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci 21, 1868-1875.

Madisen, L., Mao, T., Koch, H., Zhuo, J.M., Berenyi, A., Fujisawa, S., Hsu, Y.W., Garcia, A.J., 3rd, Gu, X., Zanella, S., Kidney, J., Gu, H., Mao, Y., Hooks, B.M., Boyden, E.S., Buzsaki, G., Ramirez, J.M., Jones, A.R., Svoboda, K., Han, X., Turner, E.E., Zeng, H., 2012. A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. Nat Neurosci 15, 793-802.

Matthews, W.B., Miller, H.G., 1972. Diseases of the nervous system. Blackwell Scientific Publications, Oxford,.

Max, M.B., Lynch, S.A., Muir, J., Shoaf, S.E., Smoller, B., Dubner, R., 1992. Effects of desipramine, amitriptyline, and fluoxetine on pain in diabetic neuropathy. N Engl J Med 326, 1250-1256.

McQuay, H.J., 2002. Neuropathic pain: evidence matters. Eur J Pain 6 Suppl A, 11-18.

Mellick, G.A., Mellicy, L.B., Mellick, L.B., 1995. Gabapentin in the management of reflex sympathetic dystrophy. J Pain Symptom Manage 10, 265-266.

Mesulam, M.M., Mufson, E.J., 1982. Insula of the old world monkey. I. Architectonics in the insulo-orbito-temporal component of the paralimbic brain. J Comp Neurol 212, 1-22.

Narita, M., Niikura, K., Nanjo-Niikura, K., Narita, M., Furuya, M., Yamashita, A., Saeki, M., Matsushima, Y., Imai, S., Shimizu, T., Asato, M., Kuzumaki, N., Okutsu, D., Miyoshi, K., Suzuki, M., Tsukiyama, Y., Konno, M., Yomiya, K., Matoba, M., Suzuki, T., 2011. Sleep disturbances in a neuropathic pain-like condition in the mouse are associated with altered GABAergic transmission in the cingulate cortex. Pain 152, 1358-1372.

Navratilova, E., Xie, J.Y., Meske, D., Qu, C., Morimura, K., Okun, A., Arakawa, N., Ossipov, M., Fields, H.L., Porreca, F., 2015. Endogenous opioid activity in the anterior cingulate cortex is required for relief of pain. J Neurosci 35, 7264-7271.

Navratilova, E., Xie, J.Y., Okun, A., Qu, C., Eyde, N., Ci, S., Ossipov, M.H., King, T., Fields, H.L., Porreca, F., 2012. Pain relief produces negative reinforcement

through activation of mesolimbic reward-valuation circuitry. Proc Natl Acad Sci U S A 109, 20709-20713.

Neugebauer, V., Li, W., 2003. Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. J Neurophysiol 89, 716-727.

Palomero-Gallagher, N., Vogt, B.A., Schleicher, A., Mayberg, H.S., Zilles, K., 2009. Receptor architecture of human cingulate cortex: evaluation of the four-region neurobiological model. Hum Brain Mapp 30, 2336-2355.

Pastoriza, L.N., Morrow, T.J., Casey, K.L., 1996. Medial frontal cortex lesions selectively attenuate the hot plate response: possible nocifensive apraxia in the rat. Pain 64, 11-17.

Patel, S., Naeem, S., Kesingland, A., Froestl, W., Capogna, M., Urban, L., Fox, A., 2001. The effects of GABA(B) agonists and gabapentin on mechanical hyperalgesia in models of neuropathic and inflammatory pain in the rat. Pain 90, 217-226.

Paxinos, G., Franklin, K.B.J., 2001. The mouse brain in stereotaxic coordinates, 2. ed. Academic Press, San Diego.

Pedersen, L.H., Scheel-Kruger, J., Blackburn-Munro, G., 2007. Amygdala GABA-A receptor involvement in mediating sensory-discriminative and affective-motivational pain responses in a rat model of peripheral nerve injury. Pain 127, 17-26.

Peyron, R., Garcia-Larrea, L., Gregoire, M.C., Convers, P., Richard, A., Lavenne, F., Barral, F.G., Mauguiere, F., Michel, D., Laurent, B., 2000. Parietal and cingulate processes in central pain. A combined positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) study of an unusual case. Pain 84, 77-87.

Ploghaus, A., Tracey, I., Gati, J.S., Clare, S., Menon, R.S., Matthews, P.M., Rawlins, J.N., 1999. Dissociating pain from its anticipation in the human brain. Science 284, 1979-1981.

Porro, C.A., Baraldi, P., Pagnoni, G., Serafini, M., Facchin, P., Maieron, M., Nichelli, P., 2002. Does anticipation of pain affect cortical nociceptive systems? J Neurosci 22, 3206-3214.

Potter, G.B., Petryniak, M.A., Shevchenko, E., McKinsey, G.L., Ekker, M., Rubenstein, J.L., 2009. Generation of Cre-transgenic mice using Dlx1/Dlx2 enhancers and their characterization in GABAergic interneurons. Mol Cell Neurosci 40, 167-186.

Qu, C., King, T., Okun, A., Lai, J., Fields, H.L., Porreca, F., 2011. Lesion of the rostral anterior cingulate cortex eliminates the aversiveness of spontaneous neuropathic pain following partial or complete axotomy. Pain 152, 1641-1648.

Rainville, P., Carrier, B., Hofbauer, R.K., Bushnell, M.C., Duncan, G.H., 1999. Dissociation of sensory and affective dimensions of pain using hypnotic modulation. Pain 82, 159-171.

Rainville, P., Duncan, G.H., Price, D.D., Carrier, B., Bushnell, M.C., 1997. Pain affect encoded in human anterior cingulate but not somatosensory cortex. Science 277, 968-971.

Rosenberg, J.M., Harrell, C., Ristic, H., Werner, R.A., de Rosayro, A.M., 1997. The effect of gabapentin on neuropathic pain. Clin J Pain 13, 251-255.

Rosner, H., Rubin, L., Kestenbaum, A., 1996. Gabapentin adjunctive therapy in neuropathic pain states. Clin J Pain 12, 56-58.

Rowbotham, M., Harden, N., Stacey, B., Bernstein, P., Magnus-Miller, L., 1998. Gabapentin for the treatment of postherpetic neuralgia: a randomized controlled trial. JAMA 280, 1837-1842.

Saper, C.B., 1985. Organization of cerebral cortical afferent systems in the rat. II. Hypothalamocortical projections. J Comp Neurol 237, 21-46.

Sawamoto, N., Honda, M., Okada, T., Hanakawa, T., Kanda, M., Fukuyama, H., Konishi, J., Shibasaki, H., 2000. Expectation of pain enhances responses to nonpainful somatosensory stimulation in the anterior cingulate cortex and parietal operculum/posterior insula: an event-related functional magnetic resonance imaging study. J Neurosci 20, 7438-7445.

Seminowicz, D.A., Laferriere, A.L., Millecamps, M., Yu, J.S., Coderre, T.J., Bushnell, M.C., 2009. MRI structural brain changes associated with sensory and emotional function in a rat model of long-term neuropathic pain. Neuroimage 47, 1007-1014.

Shi, C., Davis, M., 1999. Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. J Neurosci 19, 420-430.

Shields, S.D., Eckert, W.A., 3rd, Basbaum, A.I., 2003. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. J Pain 4, 465-470.

Shimoyama, N., Shimoyama, M., Davis, A.M., Inturrisi, C.E., Elliott, K.J., 1997. Spinal gabapentin is antinociceptive in the rat formalin test. Neurosci Lett 222, 65-67.

Sikes, R.W., Vogt, B.A., 1992. Nociceptive neurons in area 24 of rabbit cingulate cortex. J Neurophysiol 68, 1720-1732.

Smith, S.B., Crager, S.E., Mogil, J.S., 2004. Paclitaxel-induced neuropathic hypersensitivity in mice: responses in 10 inbred mouse strains. Life Sci 74, 2593-2604.

Takasu, K., Honda, M., Ono, H., Tanabe, M., 2006. Spinal alpha(2)-adrenergic and muscarinic receptors and the NO release cascade mediate supraspinally produced effectiveness of gabapentin at decreasing mechanical hypersensitivity in mice after partial nerve injury. Br J Pharmacol 148, 233-244.

Takasu, K., Ono, H., Tanabe, M., 2008. Gabapentin produces PKA-dependent pre-synaptic inhibition of GABAergic synaptic transmission in LC neurons following partial nerve injury in mice. J Neurochem 105, 933-942.

Takeuchi, Y., Takasu, K., Honda, M., Ono, H., Tanabe, M., 2007. Neurochemical evidence that supraspinally administered gabapentin activates the descending noradrenergic system after peripheral nerve injury. Eur J Pharmacol 556, 69-74.

Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K., Kaneko, T., 2003. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J Comp Neurol 467, 60-79.

Tanabe, M., Takasu, K., Kasuya, N., Shimizu, S., Honda, M., Ono, H., 2005. Role of descending noradrenergic system and spinal alpha2-adrenergic receptors in the effects of gabapentin on thermal and mechanical nociception after partial nerve injury in the mouse. Br J Pharmacol 144, 703-714.

Tanabe, M., Takasu, K., Takeuchi, Y., Ono, H., 2008. Pain relief by gabapentin and pregabalin via supraspinal mechanisms after peripheral nerve injury. J Neurosci Res 86, 3258-3264.

Tang, J., Ko, S., Ding, H.K., Qiu, C.S., Calejesan, A.A., Zhuo, M., 2005. Pavlovian fear memory induced by activation in the anterior cingulate cortex. Mol Pain 1, 6.

Tanimoto, S., Nakagawa, T., Yamauchi, Y., Minami, M., Satoh, M., 2003. Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. Eur J Neurosci 18, 2343-2350.

Tasker, R.R., 2001. History of lesioning for pain. Stereotact Funct Neurosurg 77, 163-165.

Urban, M.O., Ren, K., Park, K.T., Campbell, B., Anker, N., Stearns, B., Aiyar, J., Belley, M., Cohen, C., Bristow, L., 2005. Comparison of the antinociceptive profiles of gabapentin and 3-methylgabapentin in rat models of acute and persistent pain: implications for mechanism of action. J Pharmacol Exp Ther 313, 1209-1216.

Vaccarino, A.L., Melzack, R., 1989. Analgesia produced by injection of lidocaine into the anterior cingulum bundle of the rat. Pain 39, 213-219.

Van Damme, S., Crombez, G., Van Nieuwenborgh-De Wever, K., Goubert, L., 2008. Is distraction less effective when pain is threatening? An experimental investigation with the cold pressor task. Eur J Pain 12, 60-67.

Van Groen, T., Wyss, J.M., 1995. Projections from the anterodorsal and anteroventral nucleus of the thalamus to the limbic cortex in the rat. J Comp Neurol 358, 584-604.

Villemure, C., Bushnell, M.C., 2009. Mood influences supraspinal pain processing separately from attention. J Neurosci 29, 705-715.

Vogt, B.A., 2005. Pain and emotion interactions in subregions of the cingulate gyrus. Nat Rev Neurosci 6, 533-544.

Vogt, B.A., 2009. Cingulate neurobiology and disease. Oxford University Press, Oxford ; New York.

Vogt, B.A., Nimchinsky, E.A., Vogt, L.J., Hof, P.R., 1995a. Human cingulate cortex: surface features, flat maps, and cytoarchitecture. J Comp Neurol 359, 490-506.

Vogt, B.A., Paxinos, G., 2014. Cytoarchitecture of mouse and rat cingulate cortex with human homologies. Brain Struct Funct 219, 185-192.

Vogt, B.A., Wiley, R.G., Jensen, E.L., 1995b. Localization of Mu and delta opioid receptors to anterior cingulate afferents and projection neurons and input/output model of Mu regulation. Exp Neurol 135, 83-92.

Vogt, L.J., Vogt, B.A., Sikes, R.W., 1992. Limbic thalamus in rabbit: architecture, projections to cingulate cortex and distribution of muscarinic acetylcholine, GABAA, and opioid receptors. J Comp Neurol 319, 205-217.

Watkins, L.R., Wiertelak, E.P., Maier, S.F., 1993. The amygdala is necessary for the expression of conditioned but not unconditioned analgesia. Behav Neurosci 107, 402-405.

Wei, F., Zhuo, M., 2001. Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat. J Physiol 532, 823-833.

Whitehead, W.E., Palsson, O.S., 1998. Is rectal pain sensitivity a biological marker for irritable bowel syndrome: psychological influences on pain perception. Gastroenterology 115, 1263-1271.

Wiech, K., Ploner, M., Tracey, I., 2008. Neurocognitive aspects of pain perception. Trends Cogn Sci 12, 306-313.

Wonders, C.P., Anderson, S.A., 2006. The origin and specification of cortical interneurons. Nat Rev Neurosci 7, 687-696.

Xie, J.Y., Qu, C., Patwardhan, A., Ossipov, M.H., Navratilova, E., Becerra, L., Borsook, D., Porreca, F., 2014. Activation of mesocorticolimbic reward circuits for assessment of relief of ongoing pain: a potential biomarker of efficacy. Pain 155, 1659-1666.

Xu, H., Wu, L.J., Wang, H., Zhang, X., Vadakkan, K.I., Kim, S.S., Steenland, H.W., Zhuo, M., 2008. Presynaptic and postsynaptic amplifications of neuropathic pain in the anterior cingulate cortex. J Neurosci 28, 7445-7453.

Yamamura, H., Iwata, K., Tsuboi, Y., Toda, K., Kitajima, K., Shimizu, N., Nomura, H., Hibiya, J., Fujita, S., Sumino, R., 1996. Morphological and electrophysiological properties of ACCx nociceptive neurons in rats. Brain Res 735, 83-92.

Yoon, M.H., Yaksh, T.L., 1999. The effect of intrathecal gabapentin on pain behavior and hemodynamics on the formalin test in the rat. Anesth Analg 89, 434-439.

Zhuo, M., 2014. Long-term potentiation in the anterior cingulate cortex and chronic pain. Philos Trans R Soc Lond B Biol Sci 369, 20130146.

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