The role of dopaminergic modulation of the anterior cingulate cortex in chronic pain

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Table of Contents

Abstract

Chronic pain is a highly prevalent debilitating condition resulting from maladaptive changes following nerve injury or neurodegeneration. Those suffering from chronic pain experience allodynia, a pathological sensitivity to innocuous stimuli, and hyperalgesia, an increased sensitivity to painful stimuli. Opioids are potent analgesics but they lose efficacy due to tolerance and commonly result in side effects and use disorders. The current opioid epidemic shines a spotlight on the critical need for alternative treatment options with less damaging side effects. The development of novel analgesic approaches is contingent on our understanding of the dysregulated cortical circuits involved in abnormal pain processing. Symptoms of chronic pain are linked to an increase in pyramidal cell excitability in the anterior cingulate cortex (ACC), a cortical region involved in the processing of affective components of pain. Reducing ACC hyperexcitability alleviates allodynia and hyperalgesia, confirming its role in top-down modulation of pain and identifying a promising druggable pathway for the development of novel treatments for chronic pain. Modulation of ACC activity by dopamine (DA) is of particular interest in this search given the presence of a dense mesocortical dopaminergic projection and the expression of DA receptors across all cortical layers. Additionally, the high comorbidity between chronic pain and hypodopaminergic pathologies, such as Parkinson's Disease (PD) and major depression, suggests that the mesocortical dopaminergic pathway and cortical pain circuits are firmly linked. This thesis focuses on the modulatory role of DA on pyramidal cell excitability in ACC, and consequently on its role in regulating symptoms of pain in healthy and chronic conditions. We provide convincing evidence that DA, specifically acting on the dopamine D1 receptor (D1R), is a potent inhibitory neuromodulator of pyramidal excitability via two independent mechanisms: one linked to the opening of postsynaptic hyperpolarization-activated

cyclic nucleotide–gated (HCN) channels and another linked to facilitation of the presynaptic release of GABA. Furthermore, we demonstrate that D1R signaling in the ACC is required for effective pain relief and increasing supraspinal DAergic signaling, via intracortical injection of DA receptor agonists or systemic L-DOPA supplementation, decreases the cortical hyperexcitability typical of chronic pain and reduces the associated symptoms. Moreover, L-DOPA treatment attenuates pathological HCN dysfunction, a main mediator of neuronal hyperexcitability observed in neuropathic conditions. To check if the homeostatic mechanisms that regulate DA release in the ACC are perturbed following peripheral nerve injury, we used *in vivo* dual-color single fiber photometry in freely-moving mice expressing the DA sensor dLight1.1 and the red-shifted Ca2+ sensor jRCaMP1b. Our photometry results show that a) painful and rewarding stimuli downregulate and upregulate the release of DA in the ACC, respectively, and b) the onset of chronic pain potentiates the decrease of DA release in the ACC. Based on our findings, we conclude that a deficit in mesocortical signaling promotes the dysregulation of cortical circuits involved in pain processing, exacerbating symptoms of chronic pain. Further research is required to assess if other perceptions involving the modulation of DA release, such as reward and pain relief, are impacted in chronic pain. I hope this dissertation asserts the idea that modulating dopaminergic afferents to the ACC can be a viable strategy to effectively treat or prevent the establishment of cortical hyperexcitability, and affiliated symptoms, in chronic pain.

Résumé

La neurodégénérescence ou des nerfs endommagés provoquent une douleur chronique, une condition majeure hautement débilitante. Les personnes souffrant de douleurs chroniques font l'expérience d'une allodynie, ou hypersensibilité aux stimuli anodins, et d'une hyperalgésie, ou sensibilité accrue aux stimuli douloureux. Les opioïdes sont des analgésiques puissants mais la tolérance ainsi qu'une mauvaise utilisation font perdre leur efficacité. L'épidémie actuelle d'utilisation d'opioïdes met en lumière le besoin critique de traitements alternatifs ayant moins d'effets secondaires dommageables. Le développement d'une nouvelle approche analgésique est conditionnel à notre compréhension des circuits corticaux dérégulés impliqués dans le traitement d'une douleur anormale. Les symptômes de la douleur chronique sont liés à une augmentation de l'excitabilité des cellules pyramidales dans le cortex cingulaire antérieur (CCA), une région impliquée dans le traitement des composantes affectives de la douleur. Réduire l'hyperexcitabilité du CCA atténue l'allodynie et l'hyperalgésie, confirmant le rôle de modulateur descendant de la douleur et identifiant un chemin médicamenteux prometteur pour le traitement de la douleur chronique. La modulation de l'activité du CCA par la dopamine (DA) est d'un intérêt tout particulier dans cette recherche à cause de la présence d'une projection dopaminergique méso-corticale dense et de l'expression des récepteurs DA à travers toutes les couches corticales. De plus, le taux de comorbidité est élevé entre la douleur chronique et les pathologies hypo-dopaminergiques comme la maladie de Parkinson (PD) et les troubles dépressifs majeurs, ce qui semble indiquer que la voie dopaminergique méso-corticale et les circuits corticaux de la douleur sont intimement liés. Cette thèse porte sur le rôle de modulateur de la DA dans l'excitabilité des cellules pyramidales du CCA et par conséquent sur son action de régulation des symptômes de la

douleur dans des conditions saines ainsi que chroniques. Nous apportons des données convaincantes sur le sujet, à savoir que la DA en agissant spécifiquement sur le récepteur de dopamine D1 (D1R) est un puissant inhibiteur neuromodulateur de l'excitabilité pyramidale du CCA par deux mécanismes indépendants, l'un lié à l'ouverture de canaux post-synaptiques activés par l'hyperpolarisation et nucléotides cycliques (HCN) et l'autre lié à la facilitation de la libération présynaptique du GABA. Par ailleurs, nous démontrons que la signalisation D1R dans le CCA est nécessaire pour un soulagement efficace de la douleur et que l'augmentation de la signalisation DAergique supra-spinale par l'injection intra-corticale des récepteurs agonistes DA ou la supplémentation systémique en L-DOPA diminue l'hyperexcitabilité typique de la douleur chronique et réduit les symptômes associés. En outre, le traitement L-DOPA atténue la dysfonction pathologique HCN, un médiateur principal de l'hyperactivité neuronale observée dans des conditions neuropathiques. Pour vérifier que les mécanismes homéostatiques qui régulent la libération de DA dans le CCA sont perturbés suite à une lésion nerveuse périphérique, nous avons utilisé une approche photométrique en double couleur avec fibre unique *in vivo* chez des souris qui expriment le senseur DA dLight1.1 et le senseur calcique décalé vers le rouge jRCaMP1b. Nos résultats photométriques montrent: a) les stimuli douloureux ainsi que les stimuli de récompense réduisent et régulent la libération de DA dans le CCA, respectivement, et b) l'apparition de douleurs chroniques potentialise la baisse de libération de DA dans le CCA. D'après nos constatations nous tirons la conclusion qu'un déficit en signalisation dopaminergique méso-corticale favorise la dérégulation des circuits corticaux impliqués dans la régulation de perception douloureuse, exacerbant les symptômes de douleur chronique. Des recherches supplémentaires seront nécessaires pour évaluer si d'autres perceptions impliquant la modulation de libération de DA, comme la récompense et le soulagement de la douleur, sont impactées en conditions de

douleur chronique. J'espère que cette thèse permet d'avancer l'idée que moduler l'effet dopaminergique se rapportant au CCA peut-être une stratégie alternative viable pour traiter efficacement ou bien empêcher l'établissement d'hyperexcitabilité corticale et ses symptômes associés chez les patients atteints de douleur chronique.

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Contributions to Original Knowledge

This thesis aims to fill in the gaps in knowledge concerning the role of dopamine in modulating affective components of pain as well as whether its dysregulation is involved in promoting pathological cortical hyperexcitability in chronic pain conditions.

Below are the contributions to original knowledge of Chapters 2 and 3, the research articles this thesis is based on:

Chapter 2:

Lançon K, Qu C, Navratilova E, Porreca F, Séguéla P (2021) Decreased dopaminergic inhibition of pyramidal neurons in anterior cingulate cortex maintains chronic neuropathic pain. Cell Reports 37:109933. 10.1016/j.celrep.2021.109933.

This chapters characterizes the neuromodulatory effect of dopamine on pyramidal neurons in the anterior cingulate cortex in naïve and neuropathic animals. We identify a unique mechanism, relying on the D1R-mediated opening of HCN channels, that causes dopamine to inhibit pyramidal cells and decrease pain sensation via top-down pain modulation. As D1R activation is commonly excitatory, the identification of this novel mechanism could be relevant to other neurons expressing D1R. Furthermore, we show that D1R-signaling in the ACC is required for effective pain relief in animals with chronic pain. Supplementing supraspinal dopamine with L-DOPA reverse the pathological cortical hyperexcitability present in chronic pain and decreases the associated somatosensory symptoms. This chapter identifies a major indication that chronic pain might alter the release of dopamine in the ACC, facilitating the onset of the typical symptoms of chronic pain.

Chapter 3:

Lançon K and Séguéla P (2022) Chronic Pain Alters Dopamine Release in the Anterior Cingulate Cortex.

Based on the findings of the previous chapter, this chapter uses new technologies to assess the release of dopamine in the ACC following different types of stimulation. For the first time, we show proof that pain and reward bidirectionally control the release of dopamine in the anterior cingulate cortex, decreasing or increasing it, respectively. More importantly, following mice longitudinally before and after chronic pain, we show the onset of chronic pain significantly affects the release of dopamine in the ACC and this reduction in dopamine is correlated to the increase in cortical excitability. Additionally, we providence evidence of another dopaminergic mechanism that inhibits pyramidal neurons in the ACC. This mechanism relies on the presynaptic release of GABA and is independent to the mechanism identified in Chapter 2. Interestingly we find this pathway of dopaminergic inhibition is dysfunctional in neuropathic conditions, leading to a disinhibition of cortical pyramidal cells.

Author Contributions

Listed below is the contribution of each author to this thesis and the articles encompassing chapters 2 and 3:

Chapters 1 and 4:

KL wrote all sections and PS edited the text.

Chapter 2:

Lançon K, Qu C, Navratilova E, Porreca F, Séguéla P (2021) Decreased dopaminergic inhibition of pyramidal neurons in anterior cingulate cortex maintains chronic neuropathic pain. Cell Reports 37:109933. 10.1016/j.celrep.2021.109933.

KL performed all electrophysiology and behavioral experiments (excluding CPP).

CQ performed the CPP experiments.

KL and PS designed the project, EN and FP designed CPP experiments.

KL and PS wrote all sections and all authors contributed to the manuscript.

Chapter 3:

Lançon K and Séguéla P (2022) Chronic Pain Alters Dopamine Release in the Anterior Cingulate Cortex.

KL performed all experiments.

KL and PS wrote the manuscript.

KL and PS designed the project and the experiments.

Chapter 1: Introduction

Rationale

Literature Review

1.1 Mechanisms of Chronic Pain

1.2 Evolutionary Value of Chronic Pain

1.3 Supraspinal Pain Processing: Sensory and Affective Components of Pain Perception

1.4 Dysregulated Supraspinal Pain Circuits in Chronic Pain

1.5 Role of ACC in Pain Perception

1.6 Mediators of Pathological Cortical Hyperexcitability in Chronic Pain

1.7 The Role of Monoamines in Promoting Pathological ACC Hyperexcitability

1.8 The Dopaminergic System in Pain

1.9 Measuring Cell Excitability and *in vivo* Detection of Neuromodulator Release

Research Objectives

1.10 Determine DA's neuromodulatory effect in the ACC and its involvement in top-down pain processing

1.11 To investigate which experiences influence DA release in the ACC and whether they are affected by the onset of chronic pain

Rationale

Chronic pain is a major health issue in the general population without sustainable cost-effective treatment options. At least 20% of the global population is afflicted with chronic pain and this percentage is increasing (Dahlhamer et al, 2016). Every year, 1 in 10 adults are diagnosed with chronic pain (Golberg and McGee, 2011). Accounting for loss in productivity and health care expenses, chronic pain costs are higher than heart disease, cancer, and diabetes combined (Holmes 2016). Despite the high prevalence and societal costs of pain, treatment options are extremely limited and commonly result in use-disorders (Speed et al, 2018; Varrassi et al, 2010).

Opioids are commonly prescribed to treat chronic pain but are not a viable treatment option due to tolerance, addiction issues, and harmful side effects. Although dose escalation can be successful at overcoming tolerance in the short term, this fosters dependence, promotes addiction in the long term, and is not a sustainable solution (Ballantyne et al, 2008). In regard to adverse secondary side effects, opioids induce sedation, nausea, constipation, and most importantly life-threatening respiratory depression (Benyamin et al, 2008). The increase in opioid use in recent years, notably the opioid fentanyl, has led to the current opioid epidemic (Fischer et al 2018; Han et al, 2019). Tragically, 2458 deaths were opioid-related in Canada in 2016, a mortality rate comparable to that caused by motor-vehicle accidents (Health Agency of Canada 2019; Rudd et al, 2016). Given this, it is imperative that we pursue the development of alternative chronic pain treatment options that are more effective and less addictive.

In search of novel therapeutic targets for chronic pain, recent studies have shown chronic pain-induced alterations in the corticolimbic circuitry, including the mesolimbic and mesocortical dopamine pathways, the medial prefrontal cortex (mPFC), and the amygdala, are heavily involved

in aggravating symptoms of chronic pain (Taylor 2018). Reversing some of these pathological changes in the corticolimbic circuitry instigated by chronic pain has been successful in reducing the associated symptoms (Kang et al, 2015; Sellmeijer et al, 2018). In light of this, it is imperative to understand the dysregulated cortical circuitry involved in the pathological pain processing present in chronic pain.

To this end, this dissertation focuses on the role of dopamine in modulating the anterior cingulate cortex (ACC), a prefrontal cortical region involved in top-down pain modulation that is heavily dysregulated in chronic pain conditions.

Literature Review

This review provides a summary of the published literature relevant to the objectives and the research chapters of this dissertation. Special emphasis was placed on the role of the anterior cingulate cortex (ACC) in pathological pain signaling and on the impact of chronic pain on supraspinal dopaminergic pathways.

1.1 Mechanisms of Chronic Pain

Chronic pain is quite simply pain that lasts for an extended period of time (3 months in humans) and persists despite healing of the injured area (Merskey and Bogduk, 1994). Chronic pain can result from, but is not confined to: nerve injury, arthritis, migraine headaches, back pain, cancer, diabetes, fibromyalgia, and several neurodegenerative disorders (Blanchet et al, 2018; Defazio et al, 2008; Elliot et al, 1999; Vierck et al, 2006). As chronic pain can result from multiple sources, the distinct mechanisms that lead to chronic pain vary greatly. For example, cancer and fibromyalgia are both linked to a decrease in intra- and extracellular pH, activating the acid sensing receptors (ASICs) on peripheral nociceptive pathways (Chen et al, 2014; Mantyh et al, 2002). However, diabetes and nerve injury, generally result in nerve damage, or neuropathy, and are associated with neuropathic pain (Bouhassira et al, 2013, Gilron et al, 2015). Given the heterogeneity of chronic pain causes, they have been commonly separated into distinct categories: nociceptive syndromes, inflammatory pain, neuropathic pain, and dysfunctional central pain (Apkarian and Reckziegel, 2019).

Despite the varying mechanisms leading to the development of chronic pain, hyperalgesia and allodynia, the classical hallmark symptoms of chronic pain, are present in most cases (von

Hehn et al, 2012). Hyperalgesia is an increased sensitivity to painful stimuli whereas allodynia is pain resulting from previously innocuous stimuli.

The distinct molecular mechanisms that underlie hyperalgesia and allodynia in chronic pain are still heavily debated, but the general consensus is that these result from structural and neurochemical alterations in the peripheral and central nervous systems (Apkarian and Reckziegel, 2019). For example, in several models of neuropathic pain overactivation of the ATP-sensitive P2X4 receptor expressed on spinal microglia has been linked to tactile allodynia (Tsuda et al, 2003). Pharmacologically blocking P2X4 receptors decreases the tactile allodynia present in neuropathic pain (Tsuda et al, 2003). Conversely, peripheral sensitization in inflammatory pain models, such as rheumatoid arthritis, is linked to increases in pro-inflammatory cytokine signaling (e.g. interleukin-6) in the dorsal root ganglia (DRG) (Bas et al, 2016; Sebba 2021). Hyperalgesia and allodynia can also develop from diseases primarily affecting the central nervous system. Parkinsonian patients are well characterized for their exacerbated pain perception (Blanchet and Brefel-Courbon, 2018).

In short, many unique alterations in central and peripheral nociceptive pathways have been shown to contribute to symptoms of chronic pain in specific pain conditions and it would be unreasonable to discuss every single one (Kuner and Kuner, 2020). However, analyzing commonalities between different chronic pain disorders can give us clues as to which pathways are most associated with chronic pain symptoms and might indicate viable therapeutic targets for a wide array of disorders (further elaborated in section 1.4).

1.2 Evolutionary Value of Chronic Pain

The high prevalence of chronic pain following nerve injury in multiple species leads us to question its adaptive and evolutionary value. Sensitization of injured areas notifies us of tissue damage and prevents us from excessively using the injured area to allow for healing . In fact, children born with a congenital insensitivity to pain often die before reaching adult age due to not properly being alerted of damaged tissue (Daneshjou et al, 2012). A broken bone can lead to severe internal bleeding if not properly attended.

But why does some pain persist past the full recovery of the injured tissue, is this truly a maladaptive trait that provides no evolutionary advantage? To answer this question, a hallmark study performed by Crook and collaborators demonstrates that squids having undergone a sublethal injury are more likely to survive a predator encounter than naïve healthy squids (Crook et al, 2014). Furthermore, using anesthetics that attenuates the onset of nociceptive sensitization following injury occludes this survival advantage (Crook et al, 2014). The authors postulate that this increase in survival rate is due to pain-induced increases in alertness and anxiety that allows the squid to react to predators faster than their healthy counterparts (Crook et al, 2014).

Although we lack predators, this theory translates to modern humans and other mammals as well: heightened vigilance following injury decreases the risk for a subsequent injury (Lister et al, 2020; Walters et al, 2019). In this sense, chronic pain resulting from nerve injury can be evolutionarily adaptive. However, apart from indicating an underlying issue, the value of persistent pain in patients with cancer, diabetes, or major depression, for example, is difficult to identify. In many of these cases, debilitating chronic pain appears as a maladaptive pathological trait with little evolutionary value that decreases productivity, induces cognitive deficits, and worsens quality of life overall (Breivik et al, 2006).

1. 3 Supraspinal Pain Processing: Sensory and Affective Components of Pain Perception

Several brain regions have been shown to play a role in how we perceive pain and understanding how they function in healthy conditions is key to determine which are dysregulated in chronic pain conditions. Generally, pain perception can be separated into two main components: the sensory and the affective aspects. The somatic, or sensory, dimension of pain perception is correlated to activity in the ventrolateral thalamus, posterior insula, and the somatosensory cortex (Kuner and Kuner, 2020). Conversely, the affective, or emotional, dimension of pain perception is related to activity in the medial thalamus, anterior cingulate cortex (ACC), and the anterior insula (Kuner and Kuner, 2020).

During painful stimulation, nociceptive primary sensory neurons located in the dorsal root ganglia (DRG), comprising of Aδ and C fibers, innervate peripheral tissues and are responsible for relaying information from the periphery to the spinal cord (Julius and Basbaum, 2001). Activated spinal projection neurons then ascend the spinal cord using the spinothalamic tract and innervate different supraspinal brain regions which are responsible for discriminating between the sensory and affective components of pain perception. The sensory component of pain is largely dependent on spinal projections to the lateral thalamus, a relay hub that then projects to the primary and secondary somatosensory cortices (Kuner and Kuner, 2020). Conversely, spinothalamic projections to the medial and posterior thalamus, which then projects to the ACC and insular cortex, are associated with the affective component of pain perception (Wager et al, 2013). Although other brain regions such as the amygdala (BLA and CeA), parabrachial nucleus, hypothalamus, periaqueductal pray (PAG), and others, are known to play a role in both the sensory and affective components of pain, they are not the main focus of this dissertation (Kuner and Kuner, 2020).

1.4 Dysregulated Supraspinal Pain Circuits in Chronic Pain

The fact that chronic pain patients commonly report cognitive deficits and secondary hyperalgesia, or an increase in pain sensitivity in areas not affected by the injury, leads us to conclude that some of the aforementioned supraspinal pain circuits are dysfunctional (Berryman et al, 2013; Ferreira et al, 2016; Treede 2000). Functional MRI (fMRI) is a useful approach to monitor changes in brain activity following the onset of chronic pain. Brain regions that are more active have a greater metabolic demand, this consequently increases blood demand (Heeger and Ress, 2002). We can then analyze changes in blood flow, referred to as the BOLD signal, to determine which brain areas increased or decreased their activity following chronic pain.

Meta-analyses have identified multiple dysregulated brain regions implicated in pain perception that contribute to the negative symptoms of chronic pain. In particular, the anterior insular cortex, somatosensory cortices, and the ACC show significant increases in neuronal activity (Ferraro et al, 2021; Jensen et al, 2016). Furthermore, rather than looking at these regions as being anatomically distinct, we can measure correlations in brain regions that are simultaneously active, an analysis known as functional connectivity, to determine if the connectivity between two regions is enhanced following chronic pain. Recent studies indicate that the resting state functional connectivity between the ACC, PAG, amygdala, and parabrachial nucleus are all affected by chronic pain (Ferraro et al, 2021; Meeker et al, 2022).

Of all the dysregulated cortical areas in chronic pain, the ACC appears to be one of the most pathologically altered. Firstly, the ACC specifically has been identified as the most constitutively active brain region in chronic pain patients (Jensen et al, 2016). Secondly, the cognitive deficits of chronic pain patients coincide with the role of the ACC with working memory and attention (Berryman et al, 2013). Given this, it is critical to understand the role of the ACC in

top-down pain modulation, as well as to determine how chronic pain pathologically dysregulates it.

1.5 Role of ACC in Pain Perception

The ACC is a medial prefrontal cortical region involved in, but not limited to, attention and memory, reward-based learning, and the affective components of pain perception (Devinsky et al, 1995; Lenartowicz and McIntosh, 2005; Rainville et al. 1997). The role of the ACC in the affective components of pain was cemented by Rainville and collaborators. When subjects were presented with stimulus of varying degrees of unpleasantness, cerebral blood flow in the ACC, a proxy for neuronal activity, was correlated to the unpleasantness of the given stimulus (Rainville et al. 1997). To discern the role of the ACC in the affective, but not somatosensory, component of pain processing, another study used formalin-induced conditioned place aversion (CPA) in rodents (Johansen et al, 2001). After ablation of the rostral ACC, rodents still displayed normal sensory responses to formalin injection in the hind paw, but the CPA normally present was significantly reduced (Johansen et al, 2001). These results demonstrate that the ACC is involved in the unconscious negative emotional affect elicited by pain, but plays a smaller role in the acute sensory response.

The role of the ACC in modulating the somatosensory component of pain perception is not well established. Whereas some studies demonstrate the ACC has little influence on the sensory component of pain, other studies have shown corticospinal projections as well as projections from the somatosensory cortex relay sensory information to the ACC and optogenetic manipulation of ACC pyramidal neurons alters sensory nociceptive thresholds (Chen et al, 2018; Kang et al, 2015; Singh et al, 2020).

1.5.1 Cortical Organization of the ACC

The ACC is a neocortical region that can be separated into several functionally and structurally distinct cortical layers. Layer 1 (L1) is located closest to the external surface of the brain and is primarily composed of dendrites as well as axons projecting to and from other cortical regions. L1 is a relatively small layer but has been shown to play a role in modulating the activity of cortical neurons located in the deeper layers (Fan et al, 2020).

Layer 2/3 (L2/3) is predominantly involved in modulating the inputs and outputs of projection neurons in layers 5 and 6 (L5/6) (Shu et al, 2003). Although activation of L2/3 neurons typically amplifies excitatory currents in the deeper cortical layers, they can evoke inhibitory currents as well (Quiquempoix et al, 2018). L2/3 neurons have also been shown to project to other cortical areas (Gerfen et al, 2018). Unlike other cortical regions where Layer 4 (L4) is heavily involved in receiving thalamic inputs, it is atrophied in the ACC and its role is largely fulfilled by L2/3 (Allman et al, 2001).

Lastly, L5 and L6 are the prominent cortical layers producing the outputs to other cortical and subcortical regions and are largely responsible for the ACC's role in top-down pain modulation (Shi et al, 2022).

Layers 2/3 and layer 5 are largely composed of excitatory pyramidal cells which are modulated by a combination of afferent excitatory projections and local inhibitory interneurons. These are essentially the "gas" and "brakes" of the cortical circuitry, respectively.

1.5.2 Role of ACC in Top-Down Pain Modulation

Pyramidal neurons in L2/3/5/6 project to other cortical areas or subcortical regions (Shi et al, 2022). In the ACC, these pyramidal neurons project to a plethora of cortical and subcortical

regions including, but not limited to: the somatosensory cortex, the motor cortex, the PAG, the nucleus accumbens (NAc), the ventral tegmental area (VTA), the amygdala, the prelimbic and infralimbic cortices, the insular cortex, the medial thalamus, the lateral hypothalamus (LH), and the spinal cord (Calejesan et al, 2000; Chen et al, 2018; Shi et al, 2022; Zhou 2006). Multiple studies have confirmed that modulating ACC activity can bidirectionally control pain sensitivity. Ablation of the ACC, typically via lesions, reduces pain aversiveness while stimulating the ACC has been shown to increase nociceptive behaviors (Kang et al, 2015; LaGraize et al, 2014). ACC electrical stimulation increases Ca^{2+} responses and excitatory neurotransmission in the spinal cord while inducing nociceptive sensitization (Chen et al, 2018). Given this, these descending projections are thought to mediate the ACC's role in top-down pain modulation and their dysregulation in chronic neuropathic states could be key to promoting hyperalgesia and allodynia (Zhou 2006).

1.5.3 Dysregulated ACC in Chronic Pain as Therapeutic Target

As previously stated, the ACC is pathologically hyperactive in chronic pain conditions (Tan and Kuner 2021). Multiple human imaging studies have shown a significant increase in ACC activity in chronic pain (Jensen et al, 2016). We, and others, have shown this increased ACC activity is reflected in the firing properties of ACC L2/3 and L 5 pyramidal neurons, which display reduced thresholds required to elicit action potentials and increased firing properties in neuropathic conditions (Lançon et al, 2021; Cordeiro Matos et al, 2015). The hyperexcitable nature of these cortical projection neurons in chronic pain is likely dysregulating the aforementioned subcortical structures involved in top-down pain modulation, consequently leading to a negative affective state and an increased sensitivity to pain.

Interestingly, decreasing this cortical hyperexcitability in animal models has been demonstrated to reduce the classical symptoms of chronic pain (Kang et al, 2015; Sellmeijer et al, 2018). Using optogenetics, which uses genetically-encoded light-gated channels, allows us to selectively activate or inhibit a genetically-defined subpopulation of neurons (Deisseroth 2011). In line with established literature, studies show optogenetic activation of pyramidal neurons in the ACC increases pain sensitivity (Kang et al, 2015). More importantly however, inhibition of these hyperexcitable neurons reduces pain sensitivity in animal models of chronic pain (Kang et al, 2015). Additionally, transplantation of inhibitory GABAergic interneurons, reducing the excitatory drive on ACC projection neurons, has also been shown to provide pain relief in chemotherapy-induced chronic pain models (Juarez-Salinas et al, 2019).

There are several additional studies which use varying methods to either inhibit or activate ACC pyramidal cells; these studies all demonstrate that modulation of ACC pyramidal excitability has a powerful hold over pain perception and inhibiting these pathologically hyperexcitable pyramidal neurons is an effective method to decrease the exacerbated sensory perception observed in chronic pain models. Given this, it is critical to uncover what causes the pathological cortical hyperexcitability induced by chronic pain.

1.6 Mediators of Pathological Cortical Hyperexcitability in Chronic Pain

The excitability of neurons is influenced by multiple factors including, but not limited to: cellular input resistance, balance of excitatory and inhibitory inputs (E/I balance), and surface expression of receptors involved in directly mediating or indirectly modulating post-synaptic currents. A single culprit for promoting pathological cortical hyperexcitability in chronic pain has yet to be identified, but several studies state this is a complex multifaceted mechanism that appears

to involve alterations in pyramidal input resistance as well as E/I balance (Blom et al, 2014; Cordeiro Matos et al, 2015).

The input resistance of neurons plays a large role in dictating the current required to elicit action potentials. A reduction in input resistance, usually accomplished by opening channels expressed on the cell plasma membrane, decreases excitability (Kernell 1966). This is due to the leakage of charge mediated by these open channels, similar to the way a balloon that is punctured is more difficult to inflate. Conversely, an increase in input resistance, via the closing of channels expressed on the surface, insulates the cell and consequently increases excitability. Findings indicate that an increase in input resistance is a contributing factor promoting the chronic pain-induced hyperexcitability of ACC pyramidal neurons (Cordeiro Matos et al, 2015; Santello et al, 2017). In search for what causes this increase in input resistance, multiple studies, including ours, have identified a dysfunction in the open channel probability of hyperpolarization-activated cyclic nucleotide–gated (HCN) channels (Cordeiro Matos et al, 2015; Santello et al, 2017).

HCN channels are abundantly expressed on the dendrites of pyramidal cells in the ACC, in both L2/3 and L5, and play a large role in dictating cellular input resistance (Notomi et al, 2004; Poolos et al, 2002). These cationic channels are gated by the voltage potential across the cell membrane as well as the presence of intracellular cAMP (Wang et al, 2001). A decrease in membrane potential as well as in increase in cAMP in the cytoplasm increases the opening of these HCN channels. The onset of chronic pain has been shown to decrease the percentage of HCN channels that are open at physiological potentials, increasing the input resistance and excitability of ACC pyramidal neurons (Cordeiro Matos et al, 2015; Santello et al, 2017). As the membrane potential of pyramidal cells remains unchanged in neuropathic conditions, alterations in cAMP signaling is likely mediating this HCN dysfunction (Cordeiro Matos et al, 2015). cAMP is

converted from ATP via adenyl cyclase, a membrane bound enzyme that is modulated via activation of various G-protein-coupled receptors (GPCR) expressed on the cell membrane. G_s-coupled receptors are positively coupled to the activation of adenyl cyclase, increasing cAMP levels, while G_i-coupled receptors downregulate adenyl cyclase, decreasing cAMP levels. Given this, HCN dysfunction could be the result of excessive G_i-signaling or insufficient G_s-signaling. Shifts in the ratio of G_s/G_i signaling indicate changes in the neuromodulators that activate these GPCRs and is further elaborated on here in section 1.7-1.8.

E/I balance similarly plays a pivotal role in regulating the excitability of neurons and chronic pain-induced alterations in E/I balance is suspected to promote pathological hyperexcitability. E/I homeostasis is maintained via inhibitory inputs from local interneurons as well as excitatory inputs from afferent projections or other cortical excitatory pyramidal neurons (Yizhar et al, 2011). GABA is the primary neurotransmitter released from local inhibitory interneurons and causes tonic inhibition of pyramidal cells by opening post-synaptic $GABA_A$ receptors (Ferguson and Gao, 2018). Reports indicate the inhibitory drive onto pyramidal cells is decreased in chronic pain conditions, resulting in disinhibition (Blom et al, 2014). Given that transplantation of inhibitory interneurons into the ACC of neuropathic mice has been shown to produce analgesia, restoring inhibitory inputs onto pyramidal cells could be a viable strategy to decrease excitability (Juarez-Salinas et al, 2019). Nonetheless, the cause of this cortical disinhibition in neuropathic states has yet to be elucidated.

1.7 The Role of Monoamines in Promoting Pathological ACC Hyperexcitability

Monoamines are neuromodulators that play a critical role in maintaining the homeostasis of both G_s/G_i signaling and E/I balance. Serotonin, dopamine, and norepinephrine are three potent

neuromodulators that have been reported to influence the excitatory dynamics of cortical circuits involved in pain perception (Darvish-Ghane et al, 2016; Cordeiro Matos et al, 2015; Santello et al, 2017). The following sections will briefly breakdown the role of each of these monoamines in the ACC and whether disruptions in their signaling could be responsible for promoting pathological cortical hyperexcitability in chronic pain.

1.7.1 The Noradrenergic System in Pain

The locus coeruleus, located in the brainstem, is the primary source for norepinephrine (NE) in the brain and is vitally important in regulating our physiological processes relating to stress, sleep, sensory salience, arousal, pain, and more (Chandler et al, 2019). Dysregulation of NE signaling is involved in several medical conditions including ADHD, depression, and anxiety (Arnsten 2006; Ressler and Nemeroff, 2000). Moreover, stressors, such as chronic pain, increase the firing rate of locus coeruleus neurons, promoting the release of NE in several brain regions (Finlay et al, 1995). Optogenetic activation of locus coeruleus neurons has been shown to provide analgesia (Hickey et al, 2014). As NE receptors are abundantly expressed in the spinal cord, the analgesia induced via activation of locus coeruleus neurons is likely mediated at the spinal level (Baba et al, 2000).

Concerning the role of NE in neuromodulation of the ACC, our lab has reported that noradrenergic (NAergic) signaling influences pyramidal excitability in rodent ACC (Cordeiro Matos et al, 2015). Exogenous application of NE on ACC pyramidal neurons increases excitability and promotes persistent firing, a cellular substrate for working memory (Zang et al, 2013). This mechanism is reliant on the presynaptic activation of G_q-coupled α1 adrenoceptor, which facilitates the release of the excitatory neurotransmitter glutamate (Cordeiro Matos et al, 2015).

Furthermore, the postsynaptic G_i-coupled α2 adrenoceptor is expressed on dendritic spines of pyramidal neurons in the ACC and have been found to be colocalized with HCN channels (Wang et al, 2007). Accordingly, application of α2 receptor agonists on ACC pyramidal cells reduces the open channel probability of HCN channels, via downregulation of adenyl cyclase and cAMP, and increases excitability (Cordeiro Matos et al, 2015). Therefore, the increase in pyramidal excitability evoked by NE appears to involve a synergistic effect between the pre- and postsynaptic activation of α1 and α2 receptors. However, NE has also been shown to induce inhibition of pyramidal neurons: the G_s-coupled β adrenoceptor is present in the ACC and selective agonists decrease pyramidal excitability via increasing the open channel probability of HCN channels (Cordeiro Matos et al, 2015).

As previously stated the firing rate of locus coeruleus neurons is increased in chronic pain and it has been reported that chronic pain induces sprouting of NAergic fibers in the ACC (Cordeiro Matos et al, 2015). Given these results, it remains unclear what the role of dysregulated cortical NAergic signaling is in promoting chronic pain. As exogenous NE is excitatory, an increase in NE in the ACC should promote hyperexcitability. Nonetheless, the functional presence of antagonistic G_i-coupled α2 and G_s-coupled β adrenoreceptors as well as the fact that activation of the locus coeruleus is analgesic in the spinal cord creates uncertainty as to the exact role of NE in pathological cortical hyperexcitability.

In medical practice, intrathecal application of clonidine, an α2 receptor agonist, has been used as a successful analgesic, especially for cesarean sections and colonic surgery (De Kock et al, 2005; Filos et al, 1992). NE reuptake inhibitors, which increase NAergic tone in the central nervous system, have also seen success at alleviating neuropathic pain and comorbid pain (Briley and Moret, 2008; Jung et al, 1997).

In conclusion, the role of cortical NE in pain perception is confounded by its peripheral effects. Nevertheless, multiple studies demonstrate NE signaling is impacted by chronic pain and given the robust effect of NE on pyramidal excitability in the ACC, it is clear that alterations in NE release will promote the dysregulation of cortical circuits involved in top-down control of pain.

1.7.2 The Serotoninergic System in Pain

Serotonin, or 5-HT, is another major monoamine involved in modulating cortical circuits and pain perception. In the central nervous system, 5-HT is produced in the dorsal and caudal raphe nuclei and its dysregulation is associated with multiple psychiatric disorders and diseases, including schizophrenia, mood disorders, depression, and autism spectrum disorder (ASD) (Geyer and Vollenweider, 2008). There are a total of 15 5-HT receptors that have been characterized to date, these fall under 7 families ranging from 5-HT1 to 5-HT7 and with the exception of the ligand-gated channel 5-HT3, are all GPCRs (Hannon and Hoyer, 2008).

Research concerning the role of 5-HT in mediating pain perception is inconsistent and has primarily been focused on descending 5-HT projections to the spinal cord (Bardin et al, 2000). Some studies show that 5-HT1 agonists, intrathecally injected in the spinal cord, increase nociception while others report analgesia (Alhaider et al, 1993; Crisp et al, 1991; Eide et al, 1991). Similar results have been published using selective agonists of other 5-HT receptor subtypes, such as 5-HT2 and 5-HT3 (Bardin et al, 2000).

Although the role of 5-HT in regulating supraspinal pain circuits remains elusive, a recent study performed by the Nevian group confirmed that 5-HT7 receptors expressed in the ACC are involved in modulating pyramidal excitability and pain (Santello et al, 2017). The 5-HT7 receptor is G_s-coupled and accordingly, Santello and collaborators report that 5-HT7 agonists promote the

opening of dendritic HCN channels which consequently decreases excitability (Santello et al, 2017). These results translate *in vivo* as they also demonstrate systemic injection of 5-HT7 agonist increase nociceptive thresholds in rodent models of neuropathic pain (Santello et al, 2017). Given these results, the authors postulate that the selective 5-HT7 agonist LP-211 could become a viable strategy to treat neuropathic pain.

Despite these results, it remains unclear how chronic pain impacts the activity of 5-HT neurons in the raphe nuclei or whether they are involved in the dysregulation of cortical circuits. A recent study has described a nerve injury-induced increase in 5-HT in the mPFC that is linked to elevated activity of dorsal raphe nuclei neurons (Ito et al, 2013). The physiological consequences of these alterations is largely unknown but they have been tied to sleep disturbances (Ito et al, 2013; Tan and Kuner, 2021)

The consensus in the neuroscience community is that 5-HT is involved in pain perception yet, similar to NE, the high diversity of 5-HT receptors present in the spinal cord and brain complicates the identification of the exact circuitry involved (Bardin 2011). Although further research is required to determine the exact physiological role of 5-HT in pain perception in both the peripheral and central nervous systems, medication aimed at regulating 5-HT or its receptors could be promising at treating chronic pain.

In fact, serotonin reuptake inhibitors (SSRIs) have seen success at attenuating pain symptoms in a wide array of disorders (Aiyer et al, 2017; Micó et al, 2006). However it is important to note that serotonin-norepinephrine reuptake inhibitors (SNRIs), which are involved in inhibiting the reuptake of both 5-HT and NE, are generally more effective (Dickinson et al, 2003; Matsuoka et al, 2012). For example, duloxetine is commonly prescribed to cancer patients and has been found to show some efficacy at alleviating pain (Matsuoka et al, 2012).

1.8 The Dopaminergic System in Pain

Dopamine (DA) is another monoaminergic neuromodulator with a strong influence on spinal and supraspinal neuronal circuits. DA is primarily produced in the ventral tegmental area (VTA) and substanstia nigra (SNc/SNr) and its role in movement, learning, reward, motivation, cognition, and goal-oriented behavior is well established (Wise 2004). Disruptions in dopaminergic (DAergic) signaling leads several well studied pathologies such as Parkinson's Disease (PD), major depressive disorder, schizophrenia, mood disorders, addiction, and more (Davis et al 1991; Diehl and Gershon, 1992). The role of DA in mediating symptoms of pain is particularly interesting as recent studies have demonstrated that chronic pain significantly alters supraspinal dopaminergic (DAergic) pathways (Serafini et al, 2020).

1.8.1 Supraspinal DAergic Pathways

There are four major supraspinal DAergic pathways with unique physiological roles: the mesolimbic, mesocortical, nigrostriatal, and the tuberoinfundibular pathways (Guzman 2015).

The mesolimbic pathway pertains to DAergic neurons projecting from the VTA to the nucleus accumbens (NAc) (Adinoff 2004). Mesolimbic DAergic signaling is predominantly associated with learning, reward, and motivation (Adinoff 2004). Disruptions in the mesolimbic pathway has been shown to lead to anhedonia, or the inability to feel pleasure, as well as decreased motivation (Willner et al, 1991).

The mesocortical pathway consists of VTA projections to the prefrontal cortex and is implicated in mediating working memory, attention, and higher-order brain functions such as complex thinking and decision making (Guzman 2015). Disturbances in mesocortical signaling is associated with poor working memory and cognitive deficits (Guzman 2015; Vijayraghavan et al, 2007).

The nigrostriatal DAergic pathway is involved in motor planning and is well characterized based on the motor deficits present in PD patients. The nigrostriatal pathway encompasses DAergic projections from the substantia nigra pars compacta (SNc) to a variety of substructures in the dorsal striatum, including the caudate nucleus and putamen (Segawa 2000). Interference in the nigrostriatal DAergic pathway often leads to movement disorders, causing tremors and dyskinesia (Guzman 2015).

Lastly, the tuberoinfundibular pathway describes DAergic projections originating from the periventricular and arcuate nuclei of the hypothalamus that innervate the median eminence. This pathway is primarily involved in regulating prolactin release from the pituitary gland (Majumdar and Mangal 2015).

Although these pathways are all implicated in critical homeostatic functions, emphasis will be placed on the mesolimbic and mesocortical pathways as they are the most relevant to this thesis.

1.8.2 The Link Between DA Disorders and Chronic Pain

Unlike NE and 5-HT, the role of DA in modulating pain perception is far less ambiguous. Hypodopaminergic disorders are well known for their high prevalence of chronic pain. Over 80% of Parkinsonian patients report non-myogenic chronic pain (Blanchet et al, 2018; Defazio et al, 2008; Djaldetti et al, 2004; Ford 2010). DAergic cells of the VTA and SN are pathologically unhealthy in PD, leading to cell death (Alexander 2004). Interestingly, this decrease in supraspinal DA is linked to increased activity in the ACC (Brefel-Courbon et al, 2005). fMRI studies show the increased activity of the ACC in PD patients is comparable to the increased ACC activity seen in chronic pain (Brefel-Courbon et al, 2005). Supplementing supraspinal DA with L-DOPA, a dopamine precursor commonly prescribed to PD patients, has been shown to reduce the

pathological hyperexcitability of the ACC as well as decreases symptoms of chronic pain in PD patients (Brefel-Courbon et al, 2005).

As with PD, major depression is also associated with impairments in midbrain DAergic signaling and chronic pain is highly prevalent in patients with this mental illness (Miller and Cano, 2009). DAergic cells in the VTA of depressed subjects display altered firing properties that is linked to a decrease in DA release along the mesolimbic pathway (Yadid and Friedman, 2008). Congruent with these findings, a report indicates as many as 40% of chronic pain patients develop depression and depression is the most prevalent mental illness in chronic pain patients (Breivik et al, 2006; Tyrer 1992). The high occurrence of chronic pain in depression, and vice versa, is a strong indicator that mesolimbic DA signaling is instrumental in tuning pain perception and could be affected by the onset of chronic pain.

In fact, it appears that any alterations in midbrain DAergic signaling inevitably impacts pain perception in humans and preclinical animal models. A recent study showed that three different animals models of PD, which relies on chemical- or genetically-induced ablation of midbrain DAergic neurons, have decreased pain thresholds (Park et al, 2015; Zhou et al, 2021). Moreover, this increased pain sensitivity appears to be functionally linked to synaptic potentiation of the ACC (Zhou et al, 2021).

Inversely to the elevated pain perception following decreases in supraspinal DAergic signaling, increases in DAergic signaling are tied to analgesia and pain relief. Mesolimbic DA signaling from the VTA to the NAc is well known for its role in reward and pain relief (Navratilova et al, 2015). DA signaling in the NAc is necessary for effective pain relief and photoactivation of NAc neurons expressing DA receptors is analgesic (Sato et al, 2022; Navratilova et al, 2015). Schizophrenia is commonly characterized as having elevated levels of DA and 5-HT in the

forebrain and pain insensitivity is a common symptom (Dworkin 1994; Laruelle et al, 1999). Deep brain stimulation (DBS) of the VTA has been proven to decrease pain associated with chronic migraines (Akram et al, 2016). Moreover, monoamine reuptake inhibitors that affect the reuptake of DA are commonly prescribed for clinical depression and have seen success at decreasing pain responses produced by acid injection (Rosenberg et al, 2013).

1.8.3 DAergic Modulation of the ACC

In the prefrontal cortex, DA is known to impact the neuronal dynamics of the ACC. The Arnsten group has repeatedly demonstrated that prefrontal DA is a robust mediator of working memory performance, a core role of the ACC, in primates (Vijayraghavan et al, 2007). The ACC receives robust DAergic input via the mesocortical pathway and expresses DA receptors across all cortical layers, on both excitatory pyramidal neurons and inhibitory interneurons (Clarkson et al, 2017; Muly et al, 1998). Findings indicate the D1-receptor (D1R), a G_s-coupled receptor, is colocalized with HCN channels on dendritic spines of pyramidal neurons in the ACC (Paspalas et al, 2012). Since NE and 5-HT GPCRs have been shown to influence the activity of HCN channels, D1R activation, which promotes increases in cAMP levels, should theoretically be inhibitory to ACC pyramidal neurons (Cordeiro Matos et al, 2015; Santello et al, 2017). This has yet to be investigated. Furthermore, reports provide evidence that the somatic knockout of D1R expression in ACC significantly decreases nociceptive thresholds, indicating that D1R signaling is involved in top-down pain modulation (Darvish-Ghane et al, 2020).

If DA inhibits pyramidal cells in the ACC, a decrease in mesocortical DAergic input to the ACC should hypothetically increase pyramidal excitability via closing of dendritic HCN channels and increased input resistance. Given this, it is critical to understand whether pain influences

midbrain DAergic neurons and if so, whether dysregulation of supraspinal DA contributes to pathological cortical hyperexcitability and associated symptoms in chronic pain.

1.8.4 Impact of Chronic Pain on DAergic Pathways

Pain is well known to decrease the activity of midbrain DAergic neurons. Recent reports indicate that acute and tonic pain are both responsible for inhibiting DAergic neurons in the VTA and inducing a hypodopaminergic tone in the NAc, respectively (Gee et al, 2020; Yang et al, 2021). Activation of nociceptive fibers in the periphery via noxious stimuli inhibits VTA DAergic cells via feedforward inhibition from the lateral parabrachial nucleus (LPB) and SNr (Yang et al, 2021). Blocking LPB projections to the SNr, as a way to occlude pain-induced DA decreases in the midbrain, has been shown to abolish pain responses in rodents (Yang et al, 2021). Based on this information, chronic pain stimulation induced via peripheral nerve injury could be producing a hypodopaminergic tone in the brain. In fact, chronic pain has been shown to impair the mesocorticolimbic connectivity, and this impairment seems key to exacerbated pain sensitivity in lower back pain (Yu et al, 2020). Accordingly, evidence shows that VTA DAergic neurons are significantly inhibited in neuropathic pain models (Huang et al, 2019; Ko et al, 2018). A chronic pain-induced hypodopaminergic tone in the brain is now a widely accepted hypothesis in the neuroscience community (Taylor et al, 2016).

This hypodopaminergic hypothesis also fits with the comorbid expression of depression, anhedonia, and decreased rewards in chronic pain patients (Geha et al, 2014; Karoly et al, 2014; Munn et al, 2015). As these symptoms are strongly correlated to disruptions in mesolimbic DA signaling, chronic pain is likely causing pathological alterations in midbrain DAergic neurons (Wise 2004).

In conclusion, the prevalence of chronic pain and ACC hyperexcitability in pathologies affecting supraspinal DAergic pathways is a clear indicator that the mesocortical DAergic pathways and cortical pain circuits are firmly linked, making DA a key suspect in promoting cortical hyperexcitability in chronic pain. Multiple lines of evidence have shown that pain significantly effects DA release in the brain and despite the well-established role of the ACC in pain perception, the effects of DA, or D1R activation, on pyramidal excitability in the ACC and top-down pain modulation has not been investigated.

1.9 Measuring Cell Excitability and *in vivo* Detection of Neuromodulator Release

Neuromodulators such as DA, 5-HT, and NE have been shown to influence the excitability of cortical circuits and alterations in their release has been associated with pathological pain signaling. As this dissertation aims to determine if pathological DA signaling is involved in mediating pyramidal excitability in the ACC in chronic pain, it is critical to identify effective methods to measure pyramidal cell excitability and quantify changes in the cortical release of dopamine, *in vivo*. Below is a brief summary of the modern techniques used in Chapter 2 and Chapter 3 to fulfill this aim.

1.9.1 Whole-Cell Patch Clamp Electrophysiology

Objective measurements of the electrical properties of neurons (e.g. firing frequency, rheobase, input resistance, etc..) are commonly accomplished using whole-cell patch-clamp electrophysiology *in vitro*. With this technique, the brain is swiftly extracted from mice and placed in an oxygenated solution that mimics cerebrospinal fluid (aCSF). Using a microscope, an electrode is carefully inserted through the cell membranes of the target cell. This gives us the

capacity to measure native currents mediated by ion channels on a sub-millisecond resolution (Wu et al, 2022). Adding signaling molecules, such as DA, to the extracellular fluid allows us to determine how they affect the electrical responses of these neurons. This is a fundamental technique that is commonly used to quantify the effects of various bioactive ligands on post-synaptic currents mediated by channels, the signaling cascades involved, and consequently their effects on cellular excitability.

1.9.2 Genetically-Encoded Biosensors

Recent advances in optogenetics and genetically-encoded neuromodulator indicators (GENIs) and calcium indicators (GECIs) gives us an unparalleled opportunity to measure the effects of photoactivation of genetically defined groups on neurons on behavior and observe the release of neuromodulators *in vivo* in real time. Biosensors, or indicators, are engineered proteins that fluoresce when a desired molecule is bound to its structure. For example, GCaMPs are GFP-derived GECIs that fluoresces when in the presence of elevated levels of intracellular Ca^{2+}ions. When GCaMPs are expressed in neurons, their fluorescence can be measured to assess the relative calcium dynamics of neurons (Tian et al, 2012). As Ca^{2+} levels are positively correlated to neuronal activity, GECIs are commonly used to measure neuronal firing following behavioral or chemical stimulation. Since distinct neuronal populations express different genes, we can restrict the expression of GENIs or GECIS to specific cell populations. For example, Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), selectively expressed on excitatory pyramidal neurons, is commonly used to restrict the expression of GECIs to pyramidal neurons.

Recent breakthroughs in biosensor design have led to the development of GENIs that detect selectively the release of various neuromodulators/neurotransmitters. This includes

glutamate (iGluSnFR), GABA (iGABASnFR), acetylcholine ($GRAB_{Ach2.0}$), dopamine ($GRAB_{DA}$ and dLight), norepinephrine ($GRAB_{NE}$), serotonin ($GRAB_{5\text{-}HT}$), cannabinoids ($GRAB_{eCM}$), ATP (iATPSnFR) and opioids (dynorphin: Klight1.2, enkephalins: M-SPOTIT) (Wu et al, 2022).

As this dissertation focuses on understanding how changes in cortical DA release impacts neuronal activity, GECIs and the DA sensor dLight1.1 are extensively used in Chapter 3.

Objectives

Based on the rationale of the previous section, which critically outlines our need for non-opioidergic treatment options for pain, and the review of literature, which identifies the pathological ACC as a viable therapeutic target for chronic pain symptoms, the goal of this dissertation is to decipher the neural code for pathological pain signaling in the ACC as a means to identify targetable neuronal populations or druggable cellular pathways for effective non-opioid pain relief.

As previously mentioned in the literature review, DA is a prime suspect in promoting cortical hyperexcitability in chronic pain. The ACC expresses DA receptors across all layers and receives dense DAergic input from the VTA via the mesocortical DAergic pathway (Clarkson et al, 2017). Furthermore, hypodopaminergic disorders, such as Parkinson's' disease and depression, both display a high prevalence of chronic pain coupled to an increase in ACC activity (Brefel-Courbon et al, 2005; Miller et al, 2009). Increasing supraspinal DA tone has proven to decrease ACC activity and associated symptoms in PD patients (Brefel-Courbon et al, 2005). Taken together, these data strongly indicate that the mesocortical DAergic pathways control cortical pain circuits. Despite this, the role of DA in modulating ACC neuronal activity, and consequently top-down pain processing, is not established.

Moreover, which experiences, such as reward and pain, influence the release of DA in the ACC and whether they are affected by chronic pain has not been investigated. Reports indicate VTA DAergic cells are inhibited by the onset of chronic pain, yet, how this translates to DA release in the ACC remains unknown (Huang et al, 2019; Ko et al, 2018). We hypothesize that a decrease in cortical DA induced by chronic pain promotes pathological ACC hyperexcitability, promoting

the classical symptoms of chronic pain and the associated cognitive deficits. To this end, the main objectives of this dissertation will be:

1) Determine DA's neuromodulatory effect in the anterior cingulate cortex and its involvement in top-down pain processing.
2) To investigate which experiences influence DA release in the ACC and whether they are affected by the onset of chronic pain.

1.10 Objective 1

Determine DA's neuromodulatory effect in the anterior cingulate cortex and its involvement in top-down pain processing.

The main goals of chapter 2 will be to determine if DA modulates pyramidal cell excitability in the ACC and if so, whether dysregulation of cortical DA is a cause of pathological cortical hyperexcitability in chronic pain.

Multiple lines of evidence point to HCN channels as a main mediator of pathological cortical hyperexcitability in neuropathic pain. Our group and others have previously reported HCN dysfunction present in the ACC of neuropathic mice (Cordeiro Matos et al, 2015; Lançon et al, 2021). In chronic pain conditions, HCN channels are pathologically closed, insulating dendrites and reducing the current necessary to evoke firing. Given that HCN channels are cAMP-gated, various monoamines acting on postsynaptic G_s- or G_i- signaling have been shown to mediate the opening of HCN channels (Cordeiro Matos et al, 2015; Santello et al, 2017). For example, norepinephrine, acting on postsynaptic G_i-coupled α2 receptors, impedes the opening of HCN channels at physiologically relevant membrane potentials and consequently increases excitability (Cordeiro Matos et al, 2015). Conversely, serotonin, acting on postsynaptic G_s-coupled 5-HT7 receptors, facilitates the open of HCN channels and decreases excitability (Santello et al, 2017). Notwithstanding the well established effects of these monoamines on HCN function, the role of DA in mediating HCN channel opening has yet to be investigated.

G_s-coupled D1-like and G_i-coupled D2-like receptors are expressed across all cortical layers in the ACC (Clarkson et al, 2017). D1 receptors specifically have a higher levels of expression in layers 2/3 and 5 of the mPFC/ACC and they are colocalized with HCN channels on dendritic spines of pyramidal cells (Paspalas et al, 2012). Given this, it is important to determine

if D1 receptor activation influences I_h, the current flowing through HCN channels, and subsequently neuronal excitability. Likewise, determining if D2 receptor activation inversely mediates HCN channels is key to understanding how DA dynamically influences ACC excitability. If D1R activation is found to facilitate the opening of post synaptic HCN channels, this would indicate a deficit in cortical DA could be driving the HCN dysfunction present in neuropathic conditions. Conversely, if D2R activation is found to inhibit HCN opening, an increase in cortical DA could be promoting HCN dysfunction in neuropathic conditions.

As mentioned in the literature review, there is evidence DA influences neuronal activity in the ACC, as increasing or decreasing cortical DA significantly impacts working memory in primates (Vijayraghava, et al, 2007). However, whether DA influences the pain circuits in the ACC, leading to top-down pain modulation, has yet to be investigated. As previous studies have demonstrated inhibition of ACC pyramidal neurons can drive analgesia in chronic pain, we aim to identify if cortical injection of DA agonists can likewise modulate nociception (Kang et al, 2015; Sellmeijer et al, 2018). These experiments will be instrumental in determining if the mesocortical DA pathway can be used to effectively treat symptoms of chronic pain.

If DA is found to significantly affect pyramidal excitability in the ACC and intracortical DA injection influences nociception, our next step will be to investigate whether increasing cortical DA can attenuate symptoms of chronic pain. L-DOPA decreases ACC activity and pain symptoms in Parkinsonian patients (Brefel-Courbon et al, 2005). In light of this, we aim to investigate if L-DOPA supplementation can also be used to modulate ACC excitability and symptoms of chronic pain in rodents. The information acquired through these experiments will indicate whether supplementing supraspinal DA can be used to reverse pathological alterations in ACC circuitry following neuropathic pain.

To summarize the main goals of objective 1:

1. Investigate whether DA and specific DA receptor activation impact pyramidal cell excitability in the ACC and if so, whether HCN channels are involved.
2. Determine if intracortical injection of DA in the ACC influences nociception.
3. Establish whether increasing supraspinal DA via L-DOPA can attenuate cortical hyperexcitability and associated symptoms in neuropathic rodents.

1.11 Objective 2

To investigate which experiences influence DA release in the ACC and whether they are affected by the onset of chronic pain.

Although Chapter 2 will investigate the role of DA in the modulation of I_h, GABAergic interneurons also play a crucial role in controlling pyramidal excitability and this should not be overlooked (Ferguson and Gao, 2018). Inhibitory interneurons in the ACC have been implicated in top-down pain modulation. Optogenetic activation of PV+ and SOM+ interneurons is analgesic and GABAergic implants in the ACC of neuropathic rodents can attenuate hyperalgesia (Juarez-Salinas et al, 2019; Kang et al, 2015). As D1Rs and D2Rs are expressed on GABAergic interneurons in ACC, it is critical to investigate the role of DA in modulating GABA release (Muly et al, 1998). Furthermore, reports indicate a synaptic uncoupling between excitatory pyramidal cells and inhibitory interneurons in neuropathic ACC, resulting in disinhibition (Blom et al, 2014). As this could influence cortical hyperexcitability, we will explore whether DA mediates GABA release from interneurons and whether this specific pathway is affected by chronic pain.

Based on these results, we will also investigate which mechanisms influence DA release in the ACC. DA release is relatively well studied in the mesolimbic pathway: rewarding stimuli increases DA release in the nucleus accumbens (NAc) and painful stimuli decreases it (Lammel et al, 2011; Yang et al, 2021). However, the effect of pain and reward on mesocortical DA release, specifically in the ACC, has yet to be investigated and will be a primary objective of Chapter 3.

As we show in objective 1, D1R signaling in the ACC is required for effective pain relief in neuropathic mice, indicating pain relief promotes the release of DA in the ACC. However, recent reports suggest that pain relief-induced DA release along the mesolimbic pathway is absent

in late stages of neuropathic injury (Kato et al, 2016). As our results from objective 1 indicate supplementing supraspinal DA attenuates the pathological cortical hyperexcitability present in neuropathic mice, chronic pain could be affecting the regulatory mechanisms controlling DA release in the ACC as well. Experiences that promote the release of DA such as reward and pain relief could be attenuated whereas those that inhibit the release of DA could be potentiated. In fact, there is evidence that cortically-projecting VTA DA neurons display lower firing frequencies in chronic pain (Huang et al, 2019). Given this, we aim to establish whether the onset of chronic neuropathic pain significantly affects DA release in the ACC.

In addition to longitudinally measuring stimuli-evoked DA release in the ACC of animals undergoing neuropathic pain, we will concomitantly record noxious stimuli-evoked calcium events in pyramidal neurons. Glutamatergic afferents from the medial thalamus projecting to the ACC mediates ascending nociceptive signaling. Analyzing nociceptive calcium events in the ACC following the onset of chronic pain will give us an unparalleled opportunity to determine at which point pathological hyperexcitability sets in and whether its onset is correlated with changes in DA signaling.

To summarize the main goals of objective 2:

1. Investigate the role of DA in modulating inhibitory interneurons in the ACC and determine whether this pathway is affected by chronic pain.
2. Using reward, pain relief, and noxious stimuli, we will determine which experiences regulate ACC DA release and whether they are perturbed following nerve injury.
3. Establish whether a reduction in cortical DA is associated with the potentiation of pain-evoked ACC calcium events following nerve injury.

Chapter 2

Decreased Dopaminergic Inhibition of Pyramidal Neurons in Anterior Cingulate Cortex Maintains Chronic Neuropathic Pain

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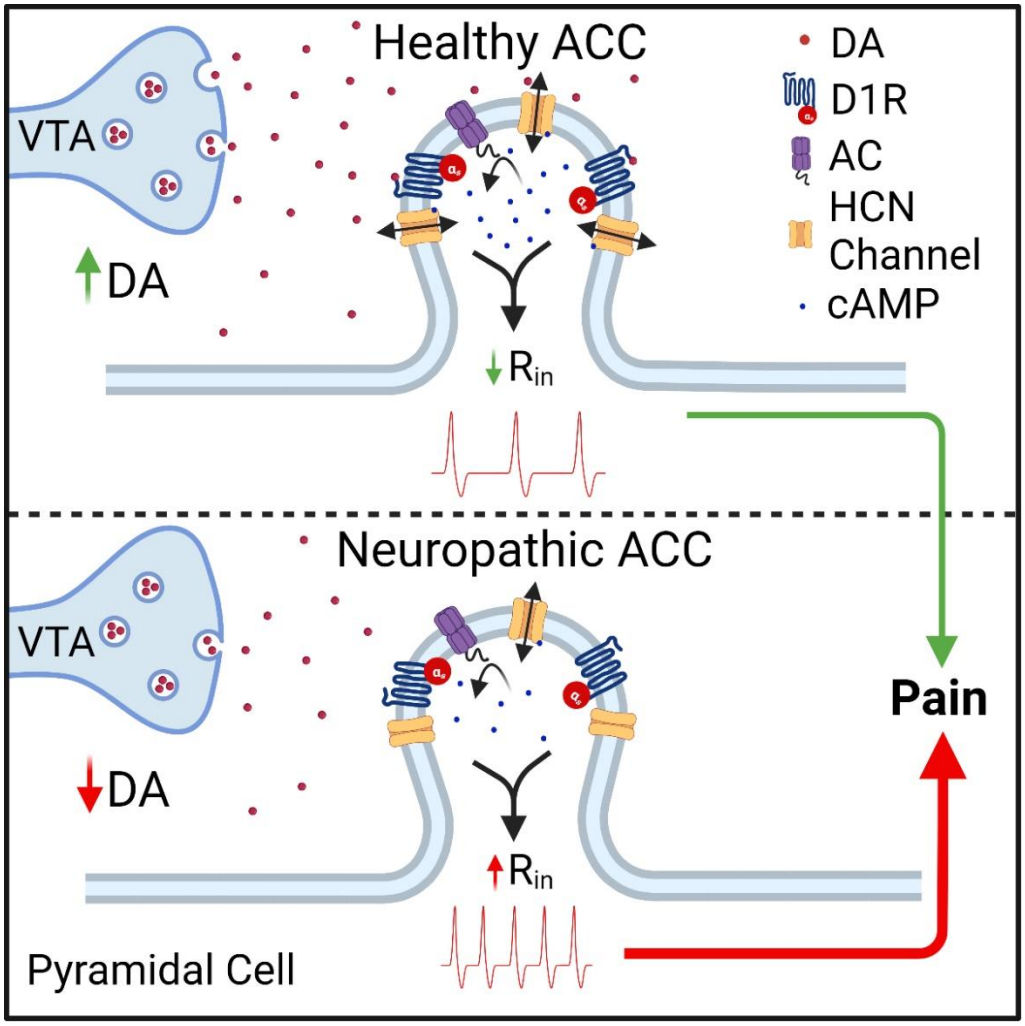
Summary

Pyramidal neurons in the anterior cingulate cortex (ACC), a prefrontal region involved in processing the affective components of pain, display hyperexcitability in chronic neuropathic pain conditions and their silencing abolishes hyperalgesia. We show that dopamine, through D1 receptor (D1R) signaling, inhibits pyramidal neurons of mouse ACC by modulation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Activation of Gs-coupled D1R by dopamine induces the opening of HCN channels at physiological membrane potentials, driving a significant decrease in input resistance and excitability. Systemic L-DOPA in chronic neuropathic mice rescues HCN channel activity, normalizes pyramidal excitability in ACC, and blocks mechanical and thermal allodynia. Moreover, microinjection of a selective D1R agonist in the ACC relieves the aversiveness of ongoing neuropathic pain while an ACC D1R antagonist blocks gabapentin and lidocaine-evoked antinociception. We conclude that dopaminergic inhibition via D1R in ACC plays an analgesic role in physiological conditions and is decreased in chronic pain.

Keywords: prefrontal cortex, dopamine, D1 receptor, HCN channels, levodopa, top-down pain modulation, monoamines, neuromodulation, pain aversiveness, ongoing pain relief, chronic pain

Graphical Abstract



Introduction

Damage to peripheral nerves induces maladaptive changes in both peripheral and central nociceptive pathways that may increase the risk of chronic pain (Campbell et al, 2006; Decosterd et al, 2000). These maladaptive changes can elicit ongoing pain as well as allodynia, a pathological sensitivity to innocuous stimuli, and hyperalgesia, an increased sensitivity to painful stimuli (von Hehn et al, 2012). These hallmark symptoms of chronic pain are linked to an increase in pyramidal cell excitability in layer 2/3 of the anterior cingulate cortex (ACC), a region of the medial prefrontal cortex (mPFC) that is implicated in the affective components of pain in both human and animal studies (Cordeiro Matos et al, 2015; Eto et al, 2011; Kamiński et al, 2017; Mhalla et al, 2010; Rainville et al, 1997). A recent meta-analysis of brain imaging data showed that the ACC is the most consistently activated region in patients suffering from chronic pain consistent with its critical role in higher-order pain processing (Jensen et al, 2016). Additionally, in experimental rodent models of inflammatory and neuropathic pain, optogenetic inhibition of hyperexcitable pyramidal cells has been reported to produce analgesic effects including decreased tactile allodynia and conditioned place preference, respectively (Kang et al, 2015; Sellmeijer et al, 2018). Collectively, these findings support the conclusion that the ACC is a region involved in regulatory top-down pain processing. Despite these findings, very little is known about how pyramidal neurons in the ACC are modulated in physiological or pathological states.

Hyperpolarization-activated cyclic nucleotide gated (HCN) channels control the excitability of many central neurons by regulating input resistance (Poolos et al, 2002). These cAMP-gated cation channels are highly expressed in the mPFC and have decreased open channel probabilities at physiological membrane potentials in chronic pain conditions (Cordeiro Matos et al, 2015; Notomi et al, 2004; Santello et al, 2017). This reduction in HCN-mediated currents (I_h) results in increased

input resistance and therefore excitability (Cordeiro Matos et al, 2015; Santello et al, 2017). Recent findings indicate that HCN channels are colocalized with G_s-coupled D1 dopamine (DA) receptors on dendritic spines of mPFC pyramidal neurons (Paspalas et al, 2013; Stoof et al, 1981).

DAergic modulation of the ACC is particularly interesting given the known role of the other major monoamines including norepinephrine and serotonin in prefrontal modulation in chronic pain states (Cordeiro Matos et al, 2015; Santello et al, 2017). In view of the well-documented effects that both norepinephrine and serotonin have on HCN channel function in the ACC and consequently on pathological cortical excitability, we hypothesized that DA, through postsynaptic D1 receptors and opening of HCN channels, can decrease pyramidal excitability and consequently influence top-down pain pathways by inhibition of pyramidal cells.

In the present study, we investigated the neuromodulatory effects of DA on intrinsic cellular excitability of pyramidal cells in the ACC and we provide electrophysiological evidence of a functional interaction between D1 receptors and HCN channels. We also examined whether an increase in DAergic input to the ACC can reduce the pathological cellular dysfunction of the ACC observed in neuropathic conditions and alleviate the symptoms of chronic pain. We found that a tonic increase in cortical DA, via long-term systemic administration of L-DOPA/carbidopa, relieves neuropathy-induced allodynia while acute intracortical injection of a selective D1R agonist drives conditioned place preference selectively in neuropathic animals indicating the relief of the aversive state of ongoing pain. Furthermore, we provide evidence that pain relief evoked by gabapentin and lidocaine also requires D1R signaling in the ACC.

Results

DAergic Inhibition of Pyramidal Cells in ACC

Following application of DA (10-50 µM) by perfusion for 15 minutes, 50% (n = 9 of 18) and 54% (n = 7 of 13) of pyramidal cells in layer II/III of the ACC were inhibited relative to baseline (data not shown).The dichotomy in response to DA between responsive and non-responsive cells was obvious, as responsive cells showed a marked inhibition of -40.1 ± 10.1% in contrast to non-responsive cells which expressed a +6.9 ± 4.5% excitation relative to baseline values following 50 µM DA application (Figure 1A) (n = 18, unpaired t-test: $t(14)=4.6$, $p = 0.0004$). Criteria for responsiveness was based on the presence of a reversible decrease in excitability. Analysis of responsive cells showed a dose-dependent inhibition. We observed that 10 µM DA reduced excitability to 84.9 ± 6.4% of baseline (15% inhibition) while 50 µM DA inhibited cells to 59.7 ± 8.4% of baseline (40% inhibition, Figure 1B and 1C) (n = 7-9; one-way ANOVA: $F(2, 21) = 7.6$, $p = 0.0032$). The inhibitory effects of DA were reversible following a 10-minute washout in all responsive cells, with excitability values for both 10 µM and 50 µM DA treatments returning to within 1.6% of control values (Figure 1C).

The input-output plot illustrates the inhibitory effect of DA on pyramidal firing activity (Figure 1C). Interestingly, DA induces an increase in the threshold required to trigger an action potential at low stimulation intensities. This is represented in Figure 1C where weak stimulations below 50 pA no longer elicited action potentials in cells treated with 50 µM DA (n = 7, 9; two-way ANOVA: $F(14, 270) = 56.0$, $p = 0.0001$). This is further demonstrated by analyzing the current required to evoke an action potential. We found DA increased the current required to evoke an action potential at -60 mV from ≈ 40 pA to 56.7 ± 8.8 (10 µM) and 68.9 ± 10.6 pA (50 µM) (n = 9, paired t-test: $t(8) = 3.5$, $p = 0.0101$)(Figure 1C). In pyramidal cells treated exclusively with aCSF alone we did

not notice any significant change in excitability (+2.0 ± 6.1% relative to baseline values, n=8, Figure 1C).

In concordance with the decreased excitability of layer 2/3 pyramidal cells of the ACC following DA application, we also observed a significant decrease in the input resistance of DA-responsive cells (Figure 1D). Input resistance for cells treated with aCSF alone was found to be 145.7 ± 8.7 MΩ whereas in the presence of 50 µM DA we measured an input resistance of 114.4 ± 10.2 MΩ (Figure 1D) (n = 8, paired t-test: t(7)=2.3, p = 0.028). Similarly, 10 µM DA also induced a trend of reduction in input resistance, however the difference was not found significant (154.8 ± 9.8 MΩ to 128.1 ± 8.7 MΩ) (data not shown).

These electrophysiological results indicate that the neuromodulator DA induces a robust and reversible inhibition of pyramidal cells in the mouse ACC, by inducing a decrease in their input resistance.

DAergic Inhibition of Pyramidal Cells Mediated by D1Rs

To determine which receptor DA acts at on pyramidal cells, we tested the effects of the selective D1R agonist SKF81297. Application of 10 µM SKF81297 induced a strong, reversible inhibition of layer 2/3 pyramidal cells in the ACC (Figure 2A), similar to the effects produced by 50 µM DA (Figure 1C). SKF81297 elicited a significant decrease in excitability relative to control values (Figure 2B) (n = 9; control: 98.0 ± 6.1 %, SKF: 68.9 ± 3.2% relative to baseline; one-way ANOVA: $F(2, 24) = 16.2$, $p = 0.0013$). SKF81297 also evoked a significant decrease in input resistance (Figures 2C) (n = 9; baseline: 151.2 ± 6.5 MΩ, SKF: 134.7 ± 9.2 MΩ; paired t-test: $t(8) = 2.17$, $p = 0.031$).

We also investigated the effects of SCH23390, a selective D1R antagonist, on the inhibitory effects of DA. Addition of SCH23390 by pre-incubation and co-application blocks both the inhibitory effect of 50 µM DA (Figure 2A) and the DA-induced decrease in input resistance (Figure 2C). Group analysis further confirms this antagonism as SCH23390 application completely abolishes the effects of DA on excitability (n = 10, +7.6 ± 4.9 % excitability relative to baseline) and on input resistance (n = 10, 166.0 ± 12.8 MΩ to 154.0 ± 13.9 MΩ following DA application) (Figures 2B and 2C). Interestingly, directly blocking D1Rs and adenylate cyclase activation via the antagonist SCH23390 caused an increase in baseline excitability (Figure 2B) (n = 10, p = 0.0004), suggesting the presence of basal dopamine tone in ACC.

We then investigated the effect of activating G_i-coupled D2-like receptors on pyramidal cell excitability (Tsu et al, 1996). We applied selective D2-like agonist quinpirole (10 µM) to pyramidal neurons in layer 2/3 of the ACC and observed that D2-like receptor activation increases their firing activity (Figure 2A). Following quinpirole application we found a significant increase in excitability in comparison to aCSF controls (Figure 2B) (n = 6, 8; repeated measure two-way ANOVA with Dunnett's multiple comparison: $F(3, 450) = 30.2$, $p = 0.0448$). Altogether, these findings indicate that postsynaptic DAergic inhibition of pyramidal cells in the ACC exclusively depends on D1R and does not involve excitatory D2-like receptors.

DAergic Modulation of Persistent Firing in ACC

Pyramidal cells in the ACC display persistent firing properties that make them capable of holding a working memory trace (Kamiński et al, 2017). Persistent firing was induced by coincident depolarization and stimulation with the mGluR1/5 agonist DHPG (10 µM). DHPG alone caused an increase of 91.5 ± 46.4% in persistent firing frequency relative to baseline (n = 11) (Figure 2D). In a large majority of persistent firing cells, application of DA (10 µM) or the D1R agonist

SKF81297 (10 μM) produced a significant decrease in firing frequency or even termination of the prolonged firing (n = 12 of 17 for DA, n = 13 of 18 for SKF81297, Figure 2D). SKF81297 and DA application induced a 61.8 ± 10.3% and 65.4 ± 6.9% reduction in persistent firing intensity, respectively, relative to baseline values (n = 12-13; one-way ANOVA: $F(2, 30) = 15.3$, $p < 0.0001$) (Figure 2D).

HCN Channels Mediate DAergic Modulation of ACC Pyramidal Cells

To determine if HCN channels contribute to DAergic inhibitory mechanisms, we pre- and co-applied the HCN channel blocker ZD7288 alongside DA during recordings. Treatment with 10 μM ZD7288 completely abolishes DAergic inhibition in the ACC (Figure 3). Following DA application in presence of ZD7288, we noticed no decrease in either excitability (Figure 3A) or input resistance (Figure 3A and 3B). Whereas 50 μM DA alone induced a 40.3 ± 8.4% decrease in excitability (previously shown in Figure 1), co-application with ZD7288 evokes a 26.8 ± 12.4% increase in excitability (n = 8, one-way ANOVA: $F(2, 22) = 13.4$, $p = 0.0002$) (Figure 3A). Blocking HCN channels suppresses the impact of DA on input resistance (n = 7) (Figure 3B). These findings are in line with previous reports documenting the effect of ZD7288 on the input resistance of central neurons (Surges et al, 2004).

To gauge the importance of HCN in maintaining the homeostatic excitability of pyramidal cells in the ACC, we tested the effect of ZD7288 alone, i.e. in the absence of DA. Application of ZD7288 dramatically increases both pyramidal excitability and input resistance to levels observed in neuropathic conditions (n = 8, un-paired t-tests: $t(7) = 3.3, 2.5$ and $p = 0.0053, 0.0245$)(Figures 3C and 7A).

We found that application of DA or SKF81297 caused a measurable increase in the resting membrane potentials (RMP) of pyramidal cells (n= 17, 15; paired t-test: $t(16) = 6.9$ and $t(14) = 8.4$, $p < 0.0001$ and $p < 0.0001$) (Figure 3D). This significant shift in RMP was not present when either D1Rs or HCN channels were blocked (n = 9, 7, 7) (Figure 3D). Given the nature of HCN channels, the depolarizing shift in RMP could be due to increasing inward cationic currents.

Although these results indicate that HCN channels expression and availability are critical for DAergic inhibition, they do not prove a functional connection between DAergic modulation and HCN channel-mediated currents (I_h). To determine if DA is involved in mediating I_h , we used voltage-clamp electrophysiology to record HCN currents, in presence of TTX and $BaCl_2$ to block voltage-dependent Na and K_{ir} channels.

As expected, our recordings indicate that application of 50 µM DA does not cause any significant changes in maximal I_h currents measured at -130 mV. However, we noticed a significant increase in I_h around physiological membrane potentials (ex. -70 mV) (Figure 4A). A large population (64.2%) of pyramidal neurons in the ACC showed an increase in I_h in response to DA (n = 9 of 14). At -70 mV, I_h increases from 18.4 ± 2.7 pA to 28.0 ± 3.1 pA following application of 50 µM DA (Figure 4A). This modulation of I_h was found reversible as washing-out of DA with aCSF for 10 minutes brings back I_h to baseline values (n = 10; paired t-test: $t(9) = 3.8$, $p = 0.0022$).

A voltage-dependent increase in I_h is associated with an increase in fractional activation of HCN channels rather than in the number of HCN channels present at the cell surface (Biel et al, 2009). To check if DA induces an increase in HCN open channel probability, we analyzed HCN tail currents before and after DA application (Figure 4B). We found a significant increase in fractional activation of I_h (n = 5; repeated measure ANOVA: $F(9, 40) = 64.7$, $p < 0.0001$) (Figure 4B). Consequently, DA induces a rightward depolarizing shift in the half-activation potential of HCN

channels, from -89 ± 3 mV to -68 ± 8 mV (Figure 4B) (n = 5; paired t-test: t(8) = 2.5, p = 0.0390), indicating that DAergic inhibition is mediated by the modulation of HCN channel activity in pyramidal cells of the ACC.

To ascertain whether D1R activation also increased HCN acitvity in layer 5 pyramidal neurons we analyzed *sag* ratios before and after 10 µM SKF81297 application. Despite limitations of somatic patching in large pyramidal cells, we found a modest but significant increase in the *sag* ratio following SKF81297 application at physiological relevant potentials, from 1.11± 0.01 to 1.13 ± 0.01 (n = 16; paired t-test: t(15) = 2.4, p = 0.0317, data not shown). In line with previous results we found no change in the max *sag* ratio recorded at -100 mV, from 1.10 ± 0.01 to 1.09 ± 0.01 (n = 16; paired t-test: t(15) = 1.9, p = 0.0688).

DAergic Inhibitory Mechanisms Remain Functional in Neuropathic Conditions

To determine whether DA-mediated pyramidal inhibition is still effective in ACC in neuropathic conditions, we tested the effects of exogenous DA and SKF81297 on pyramidal excitability (following +100 pA pulse) in layers 2/3 and 5 in mice 4 weeks post-SNI surgery. We observed 50% and 33% of layer 2/3 and layer 5 ACC pyramidal neurons responding to SKF81927, respectively, values similar to the percentage of responsive cells in sham animals. SNI mice with tactile and cold allodynia displayed significant inhibition in both layers 2/3 and layer 5 in response to 50 µM DA application, similar to naïve mice (Figure 5A and 5C) (n = 4-5; paired t-test: t(4,4) = 2.65, 3.16; p = 0.038, 0.025 for layer 2/3 and 5, respectively). Additionally, 10 µM SKF81297 also decreased pyramidal excitability in both SNI and sham mice (Figure 5B and 5D) (n = 4-6; paired t-test; t(4-5) = 3.33, 5.00; p = 0.029, 0.0077; for layers 2/3 and layer 5, respectively). At +40 pA stimulation, both 50 µM DA and 10 µM SKF81297 application resulted in full silencing of responsive pyramidal neurons (data not shown). Excitability of pyramidal neurons not exposed

to DA or SKF81297 stayed within ~ 2% of baseline values in both SNI and sham mice (data not shown).

L-DOPA/Carbidopa Administration Rescues Mechanical and Cold Sensitivity in SNI mice

Our electrophysiological results, showing that the DA inhibition mechanism is preserved in neuropathic conditions, suggest that DAergic input to the ACC could be diminished in neuropathic conditions. Therefore, we tested the effects of long-term administration of the DA precursor L-DOPA, with carbidopa to prevent its peripheral metabolism, in neuropathic mice. We measured sensory thresholds in neuropathic mice using the von Frey assay and acetone cold test to measure tactile and cold allodynia, two somatosensory hallmarks of chronic neuropathic pain. Two weeks following SNI surgery, with continuous L-DOPA (75 mg/24 hr/kg) and carbidopa (10 mg/24 hr/kg) intake, we observed that treated mice did not display significant ipsilateral tactile hypersensitivity when compared to the control SNI group (n = 7-11; repeated measure two-way ANOVA with Dunnett's multiple comparisons: $p = 0.0068$) (Figure 6A). Similarly, L-DOPA/carbidopa-treated neuropathic mice did not display ipsilateral allodynic responses in the acetone cold test 3 weeks following SNI (Figure 6B).

To test whether L-DOPA is effective at reducing hyperalgesia only when provided during a critical time window following SNI surgery, we delayed the start of L-DOPA treatment in one group of mice until 2 weeks following the surgery (SNI + Delayed L-DOPA). Following two weeks on L-DOPA and 4 weeks post-SNI surgery, these mice were statistically identical to the sham, L-DOPA-treated sham and L-DOPA-treated SNI groups and significantly more antiallodynic than the control SNI group (n = 8; repeated measure two-way ANOVA with Dunnett's multiple comparisons: $p = 0.0120$) (Figure 6A). As a control, we added a group of mice who had undergone a sham surgery and supplemented their diet with L-DOPA/carbidopa. The L-DOPA-treated sham

group did not differ significantly from the non-treated sham group (n = 6, 10) (Figure 6A). Consistent with established literature, mice supplemented with L-DOPA/carbidopa displayed significant motor side-effects, including hyperactivity, starting 5 weeks following the onset of the treatment (Lundblad et al, 2005). No behavioral experiments were conducted on mice showing motor symptoms related to L-DOPA.

Daily L-DOPA/Carbidopa Administration Rescues Excitability and HCN Function in Neuropathic ACC

To assess how the behavioral characteristics of neuropathic mice translate to physiological properties of pyramidal neurons in the ACC, we analyzed the firing frequencies, input resistance, and HCN properties of layer 2/3 pyramidal neurons of SNI mice and SNI mice supplemented with L-DOPA/carbidopa. Four weeks following SNI surgery, with continuous L-DOPA (75 mg/24 hr/kg) and carbidopa (10 mg/24 hr/kg) intake, we observed that neuropathic mice did not display cortical hyperexcitability (Figure 6C). ACC pyramidal cells of L-DOPA-treated SNI mice had firing frequencies on par with sham mice, significantly lower than SNI mice not supplemented with L-DOPA. At + 100 pA stimulation, pyramidal cells from control SNI mice without L-DOPA fired at 18.0 ± 1.1 Hz whereas pyramidal cells from L-DOPA-treated SNI mice fired at 13.8 ± 0.8 Hz (Figure 6C). This effect of the L-DOPA treatment on excitability is also illustrated by the input-output plot (Figure 6C) (n= 13-27; repeated measure two-way ANOVA with Dunnett's multiple comparison: at 100 pA p= 0.0274). The impact of the L-DOPA treatment of mice on excitability of pyramidal cells in the ACC is logically reflected in changes in their input resistance (Figure 6C, from 247.8 ± 7.9 MΩ in SNI to 199.0 ± 9.7 MΩ in SNI mice supplemented with L-DOPA/carbidopa (n = 9, repeated measure two-way ANOVA with Dunnett's multiple comparison: p = 0.0025).

As HCN channel dysfunction is a major driving force causing cortical hyperexcitability in SNI mice, we also measured I_h and the fractional activation of HCN channels in neuropathic conditions with or without treatment with L-DOPA. As we have previously reported (Cordeiro Matos et al, 2015), we measured a significant shunting of pyramidal I_h in SNI mice, whereas pyramidal cells of SNI mice supplemented with L-DOPA/carbidopa did not display this downward shift, but rather displayed I_h values almost identical to those seen in sham mice across all voltages (Figure 6D) (n= 27-34; repeated measure two-way ANOVA with Dunnett's multiple comparison). This shift in I_h is mirrored in the open channel probability of HCN at physiologically relevant membrane potentials. For example, at -70 mV, 16.9 ± 2.3% of HCN channels are open in the SNI + L-DOPA group whereas only 8.8 ± 4.2% of HCN channels are open in the control SNI group (Figure 6D) (n= 25-28; repeated measure two-way ANOVA with Dunnett's multiple comparison: p= 0.07).

Daily L-DOPA/Carbidopa treatment relieves ongoing pain in SNI mice

Systemic L-DOPA/carbidopa significantly decreased tactile and thermal allodynia in SNI mice. To establish if L-DOPA/carbidopa could also produce relief of pain aversiveness, we determined if this treatment prevented gabapentin-induced conditioned place preference in SNI mice. SNI mice were pretreated with L-DOPA/carbidopa (i.p. 20 mg/10ml/kg and 4 mg/10ml/kg, per 24h) for 12 days starting immediately after SNI surgery and then at 15 days after the surgery evaluated for conditioned place preference to acute administration of gabapentin (i.p. 30 mg/ml/kg). SNI mice pretreated with saline displayed significant conditioned place preference following gabapentin (Figure 7A, n=16; paired t-test, t(9)=3.419; p=0.0076) suggesting relief of ongoing pain. In contrast, SNI mice pretreated with L-DOPA/carbidopa did not display significant preference to the chamber paired with gabapentin, suggesting that increased dopaminergic signaling produced relief of ongoing pain revealed by decreased motivation to seek pain relief

(Figure 7A, n=10; paired t-test, t(15)=0.017; p=0.987). The difference score between L-DOPA/carbidopa and saline pretreated mice was significantly different (Figure 7A, n=10-16; unpaired t-test, t(24)=2.575; p=0.0166).

Intra-ACC Injection of D1R Agonist SKF81297 Produces Conditioned Place Preference in Neuropathic Rats

Because DAergic inhibition of pyramidal cells is mediated by D1Rs, we investigated if a single administration of SKF-81297, a dopamine D1R agonist, modulates allodynia and ongoing pain in animals with neuropathic pain. To allow site specific SKF-81297 microinjection, we measured pain behavior in adult male Sprague Dawley rats with bilateral ACC cannulas and either SNI-induced chronic neuropathic pain or sham surgery. Bilateral microinjection of SKF-81297 in the ACC (0.5 µg/0.5 µl) had no effect on tactile responses in either SNI or sham rats (Figure 7B). ACC SKF81297 produced conditioned place preference only in SNI rats (Figure 7B, n=15; paired t-test; t(14)=3.431; p=0.0041). Intracortical injection of SKF81297 in sham-operated animals did not produce a chamber preference (Figure 7B, n=17; paired t-test; t(16)=0.521; p=0.609). As illustrated in Figure 7B, there is a significant difference between sham and SNI animals in CPP difference score (n = 15 – 17; unpaired t-test: t(30) = 3.06, p = 0.0047).

Relief of ongoing neuropathic or incisional pain depends on D1R Signaling in the ACC

Relief of pain is a reward that is associated with increased activity of VTA DAergic neurons and DA release in the mesolimbic pathway (Navratilova et al, 2012). As VTA DA neurons also project to the cortex, we sought to determine whether relief of pain in SNI rats elicited by gabapentin, a common treatment for neuropathic pain, is dependent on ACC D1R signaling. We therefore bilaterally microinjected the selective D1R antagonist SCH23390 (10 µg/0.5 µl) in the ACC before

gabapentin conditioning and found that while SNI rats pretreated with saline displayed a significant preference to the gabapentin paired chamber (Figure 7C, n=11; paired t-test; t(10)=2.48; p=0.033), no preference was observed in SNI rats pretreated with ACC SCH23390 (Figure 7C, n=15; paired t-test; t(14)=0.123; p=0.904).

To verify if ACC D1R signaling is also necessary for relief of post-surgical pain, we produced relief of ongoing pain with lidocaine-induced nerve block in the incisional pain model. ACC saline pretreated rats that had undergone the incisional pain procedure showed significant preference to the chamber previously paired with lidocaine injected at the popliteal fossa (Figure 7D; n=8; paired t-test; t(7)=3.14; p=0.017). However, preference to lidocaine-induced nerve block was prevented by pretreatment of SCH23390 (10 µg/0.5 µl), bilaterally injected in the ACC before conditioning (Figure 7D; n=6; paired t-test; t(5)=1.45; p=0.206). SCH23390 had no effect in sham-operated rats. There was a significant effect of treatment group when comparing CPP difference score for SCH23390/sham; saline/incision and SCH23390/incision animals (Figure 7D; n=6-8; one-way ANOVA; p=0.0114; Tukey's multiple comparison test: SCH23390/sham vs. saline/incision $p<0.05$; saline/incision vs. SCH23390/incision $p<0.05$).

Discussion

Understanding how chronic pain impacts cortical circuitry is key to the development of improved effective treatments for patients. Modulation of ACC neuronal activity by DA is of particular interest in this search given the key role of ACC in the processing of affective components of pain perception, the presence of a dense mesocortical DAergic projection, and the expression of DA receptors across all cortical layers (Clarkson et al, 2017). Our findings indicate that DA is inhibitory to a large subpopulation of pyramidal neurons in layer 2/3 and layer 5 ACC, both in physiological and neuropathic pain states. DAergic inhibition of ACC neurons raised the rheobase

by approximately 30 pA, a difference that could determine whether a neuron fires or not following pain stimuli-evoked postsynaptic potentials in the ACC *in vivo* (Sikes et al, 1992). We used selective D1R and D2-like ligands to determine that only D1Rs are involved in this inhibition. The inhibition induced by the selective D1R agonist SKF81297 mimicked the inhibition induced by DA while the selective D2-like agonist quinpirole evoked an increase in pyramidal excitability. Additionally, we show that the D1R antagonist SCH23390 antagonizes DA's inhibitory effects. Although it is arguable that these DA-evoked excitatory changes are not present *in vivo* due to the upregulation of AMPA receptors via PKA phosphorylation, DA has been shown to decrease evoked-postsynaptic potentials (EPSPs) in the ACC (Darvish-Ghane et al, 2016; Esteban et al, 2003; Sun et al, 2005).

The ACC plays a pivotal role in working memory and patients suffering from chronic pain commonly report impairments in working memory performance (Phelps et al, 2021; Schnurr et al, 1995; Bradley et al, 1989). The property of persistent firing observed in pyramidal cells of the ACC provides a cellular substrate for working memory (Kaminski et al, 2017). We used a glutamatergic model of persistent firing evoked by the mGluR agonist DHPG to test the effects of both DA and SKF81297 on the frequency of action potentials during the persistent depolarization (plateau potential). Our results indicate that SKF81297 and DA consistently decrease the duration of plateau potential and the frequency of persistent firing in the ACC. Thus, the loss of working memory performance measured in chronic pain disorders and the overall effect dopamine and D1R antagonists on working memory could be related to this strong DAergic modulation of persistent firing in the ACC (Cai et al, 1997; Bradley et al, 1989; Kamiński et al, 2017; Panegyres 2004; Sawaguchi et al, 1991).

The dependence of DAergic inhibition on G_s-coupled D1 receptors and not on G_i-coupled D2-like receptors led us to conclude that cAMP plays a pivotal role in controlling the excitability of ACC pyramidal neurons. Our group and others have shown that cAMP-gated HCN channel dysfunction plays a major role in promoting cortical hyperexcitability in neuropathic conditions (Cordeiro Matos et al, 2015; Santello et al, 2017). We conclude that the firing activity of pyramidal cells in the ACC is controlled by a balance of monoaminergic inputs through their G_s and G_i-coupled receptors with opposite effects on HCN channel function: activation of G_i-coupled α_2 adrenergic receptors shuts down HCN channels while activation of G_s-coupled 5-HT7 and D1 receptors increase the opening of HCN channels (Santello et al, 2017; Zhang et al, 2013).

D1 receptors are co-localized with HCN channels on dendritic spines of pyramidal neurons and we confirmed that DA modulates HCN channels through D1 receptors (Paspalas et al, 2013). Our results show D1 receptors induce an increase in resting membrane potential which is prevented when HCN is blocked with ZD7288, likely due to inward cationic currents mediated by HCN channels. However, opening of HCN channels also induces a decrease in input resistance stemming from a reduction in charge insulation in dendritic spines (Surges et al, 2004). Therefore, the decrease in input resistance induced by D1R activation is likely overriding the slow depolarization, resulting in inhibition. Although DA does not increase the maximal amplitude of I_h, we noticed a significant increase in HCN-mediated currents at physiological membrane potentials. Accordingly, the half-activation potential, the membrane potential at which half of HCN channels are open, decreases drastically in the presence of DA. This suggests a change in internal cAMP levels rather than a change in the number of HCN channels at the cell surface. These results are also reflected in the fractional activation of I_h at physiological membrane potentials; at -60 mV, DA approximately triples I_h fractional activation.

We provide evidence that the signaling mechanisms underlying D1R/HCN-mediated inhibition are still functional in the ACC of neuropathic animals, strongly suggesting hypoactive dopaminergic inputs. Interestingly, pathologies with hypoactive supraspinal DAergic pathways, such as Parkinson's Disease (PD) and major depression, show a high prevalence of non-myogenic chronic pain (Blanchet et al, 2018; Defazio et al, 2008; Djaldetti et al, 2004; Ford 2010). The VTA and the SNc, the main sources of mesocortical DA, display altered firing frequencies and cell death in depression and PD, respectively (Alexander 2004; Liu et al, 2018; McGeer et al, 1988). Furthermore, there is evidence linking a GABA-mediated reduction in dopaminergic activity in the VTA of rodents afflicted with chronic neuropathic pain (Huang et al, 2019; Ko et al, 2018). A reduction in cortical DA could be causing the high comorbidity of chronic pain in patients with PD as fMRI studies display pathological hyperactivity in the ACC (Brefel-Courbon et al, 2005). Administration of L-DOPA, a DA precursor that increases DA release in the brain, and carbidopa, a peripheral DOPA decarboxylase inhibitor, alleviates symptoms of chronic pain and normalizes ACC hyperactivity in patients with PD (Antinori et al, 2018; Brefel-Courbon et al, 2005; Schestasky et al, 2007). There is also increasing evidence linking other hypodopaminergic disorders, such as clinical depression, to chronic pain (Fishbain et al, 1997; Taylor et al, 2016).

To test this idea of a hypodopaminergic state in chronic pain conditions, we supplemented chronic neuropathic mice with systemic L-DOPA and carbidopa, a treatment that was successfully used to treat pain in patients with PD (Brefel-Courbon et al, 2005). Two weeks following the start of L-DOPA/carbidopa administration, mice that had undergone SNI surgery did not show the typical hypersensitivity of animals in chronic pain. Thus, tactile and cold allodynia of SNI mice treated with L-DOPA/carbidopa did not differ from sham-operated controls. Additionally, delaying the L-DOPA treatment until 2 weeks post SNI showed reversal of established tactile hypersensitivity.

Daily L-DOPA/carbidopa treatment of SNI mice also prevented development of ongoing pain indicated by lack of CPP to gabapentin that was observed in saline treated SNI mice. These data suggest that chronic neuropathic pain is associated with a hypodopaminergic state that persists for an extended period. Administration of L-DOPA both prevented and reversed established behavioral consequences of neuropathic pain suggesting important therapeutic opportunities. In fact, clinical studies are already showing promising results using L-DOPA/carbidopa to treat chronic low back pain (Reckziegel et al, 2019). Although carbidopa was present to limit the peripheral effects of L-DOPA, we confirmed these behavioral changes are consistent with a reduction in hyperexcitability and input resistance of pyramidal cells in the ACC to levels on par with sham-operated mice and are not peripherally driven. We also measured a dramatic increase in I_h at relevant membrane potentials in L-DOPA-treated SNI mice, particularly at resting potentials where there are no HCN channels open in SNI conditions.

Consistent with the demonstration of hypodopaminergic state in animals with chronic pain and with the role of D1R on the electrophysiological properties of pyramidal neurons, we observed a CPP effect in SNI rats following acute activation of D1 receptors with SKF81297 in the ACC. As the CPP paradigm reflects learning resulting from reward and we did not observe SKF81297-paired chamber preference in sham animals, our findings indicate that D1R activation provides reward associated with pain relief (King et al, 2009; Porreca et al, 2017). Lack of SKF81297-paired chamber preference in sham animals is also in agreement with our findings of no hypoalgesia in the sham L-DOPA-treated group compared to the sham control group.

Furthermore, we show that the pain relief produced by gabapentin and lidocaine nerve block, treatments that are known to be analgesic in humans, depends on D1R signaling in the ACC. Gabapentin pain relief is associated with activation of VTA DAergic neurons and subsequent

release of DA in the mesolimbic pathway (Navratilova et al, 2012). Bilateral injection of D1R antagonist SCH23390 in the ACC prevented the conditioned place preference induced by gabapentin in a model of neuropathic pain as well as the pain relief induced by a peripheral nerve block with lidocaine in a model of post-surgical pain. As L-DOPA/carbidopa treatment prevents gabapentin-induced CPP in SNI mice, we suggest that the pain relief evoked by opioids and other non-opioid analgesic drugs rely on increasing cortical DA levels (Johnson et al, 1992).

Although it remains unclear whether hyperexcitability in the ACC is a cause or effect of chronic pain state, increasing evidence suggests inhibiting pyramidal cells of the ACC induces analgesia (Kang et al, 2015; Kuner et al, 2017; Sellmeijer et al, 2018). Our findings, in two different species, strongly suggest that DA is a major inhibitory neuromodulator of pyramidal activity in the ACC in pathological pain states. We propose that dysregulation of this DAergic inhibition contributes to the expression of affective and somatosensory features typical of chronic pain, and normalization with D1R agonists may provide significant therapeutic benefit.

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Contributions: Experiments were performed by KL and CQ. KL and PS designed the project, EN and FP designed CPP experiments, and all authors wrote the manuscript.

Declaration of Interests: None to report.

Figures and Legends

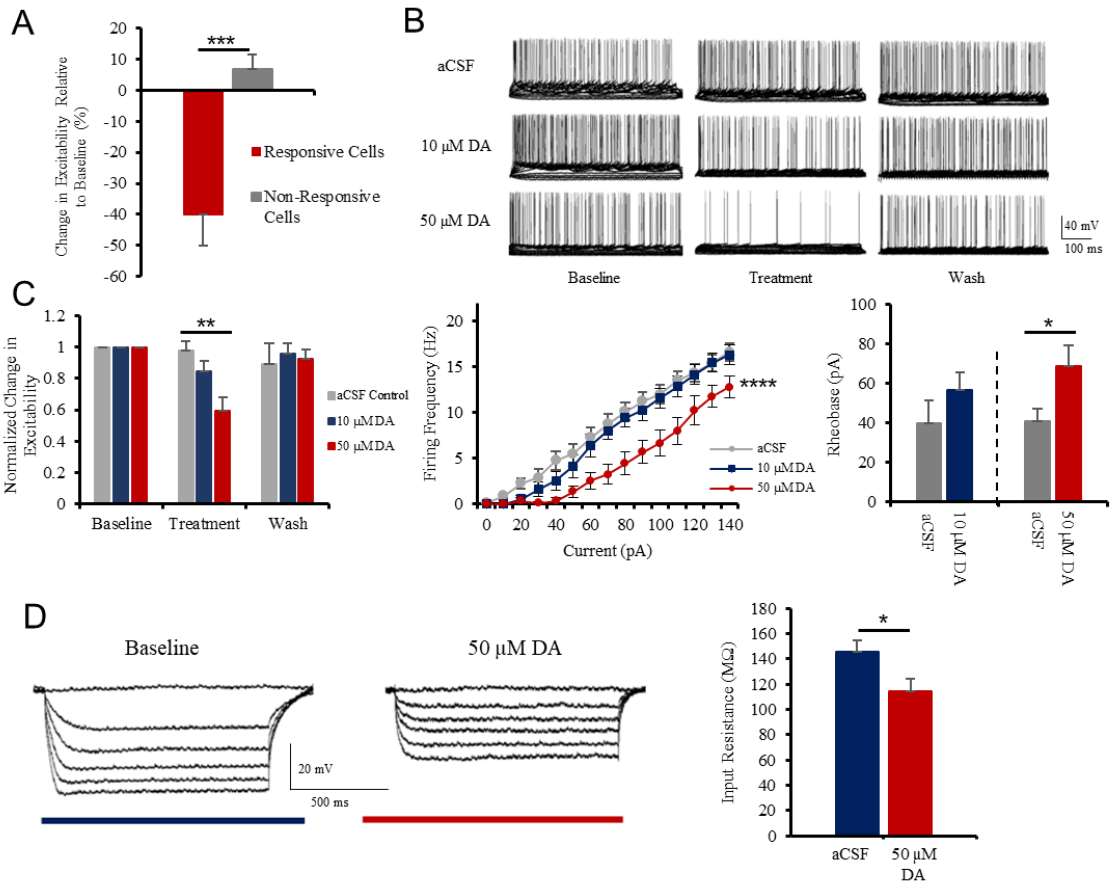

Figure 1. DA inhibits pyramidal cells in layer 2/3 of the mouse ACC.

A. Following application of 50 µM DA, a large subset of layer 2/3 pyramidal neurons in the ACC shows strong inhibition (n = 18).

B. 10-50 µM DA reduces excitability in a dose-dependent and reversible fashion, compared to control conditions, electrophysiological current-clamp traces shown.

C. Left panel: Quantitative group analysis shows that 50 µM DA significantly inhibits pyramidal firing activity in the ACC in comparison to control conditions. Middle panel: Input-output plot demonstrates interaction between stimulation intensity and firing frequency following treatment

with DA. Right panel: Application of 50 μM DA induces a significant increase in pyramidal rheobase (n = 7-9).

D. Left panel: Typical current-clamp recordings illustrating alterations in input resistance following treatment with 50 μM DA. Right panel: Quantitative group analysis of input resistance of pyramidal cells treated with aCSF or 50 μM DA (n = 8).

Values represented as means ± SEM: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

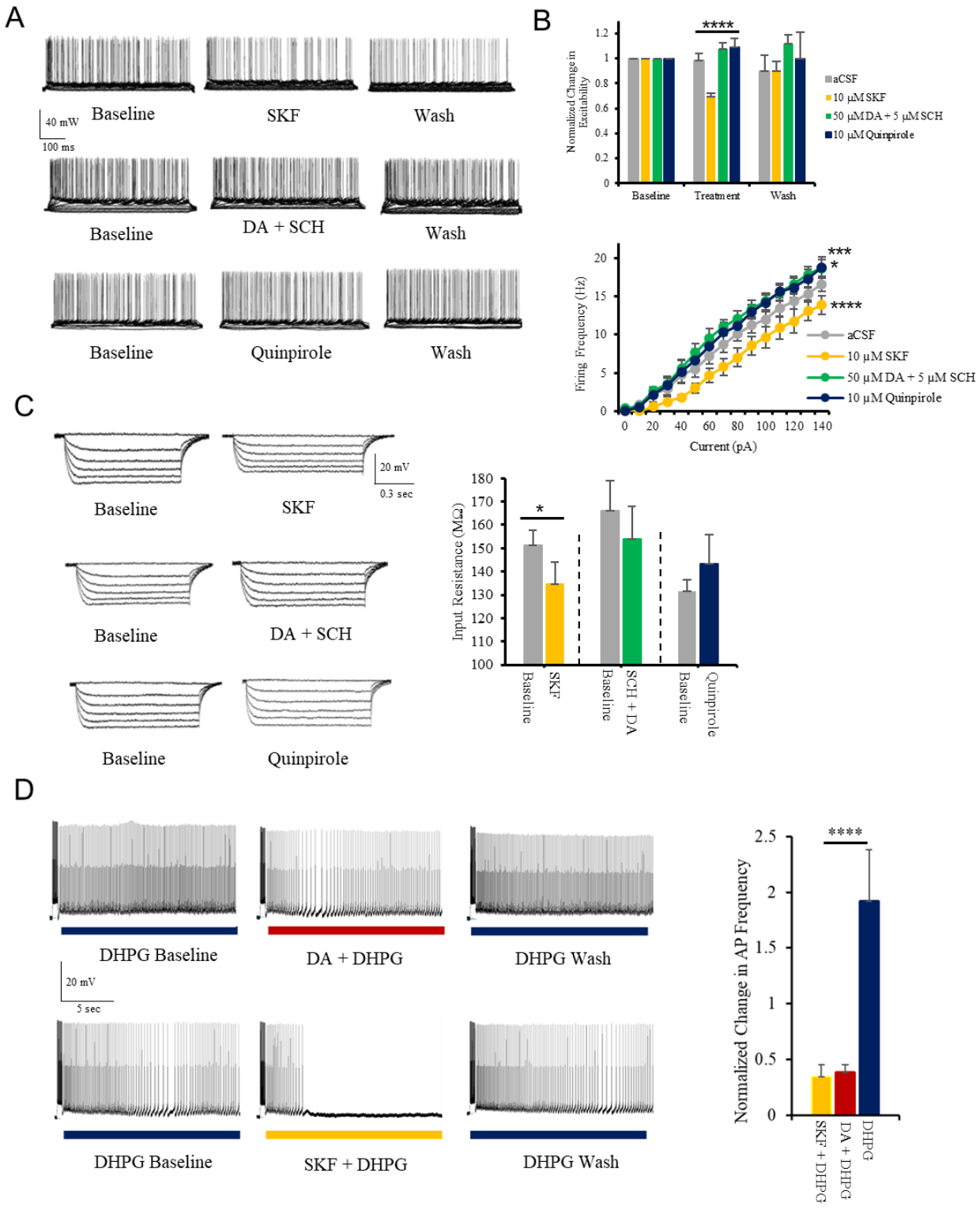
A
40 mW
100 ms
Baseline
SKF
Wash
Baseline
DA + SCH
Wash
Baseline
Quinpirole
Wash
B
Normalized Change in Excitability
Baseline
Treatment
Wash
****
aCSF
10 µM SKF
50 µM DA + 5 µM SCH
10 µM Quinpirole
Firing Frequency (Hz)
Current (pA)
***
*
****
C
20 mV
0.3 sec
Baseline
SKF
Baseline
DA + SCH
Baseline
Quinpirole
Input Resistance (MΩ)
*
Baseline
SKF
Baseline
SCH + DA
Baseline
Quinpirole
D
DHPG Baseline
DA + DHPG
DHPG Wash
20 mV
5 sec
DHPG Baseline
SKF + DHPG
DHPG Wash
Normalized Change in AP Frequency
****
SKF + DHPG
DA + DHPG
DHPG

Figure 2. DAergic inhibition of pyramidal cells in ACC is exclusively mediated by D1 receptors.

A. Typical current-clamp recordings illustrating alterations in pyramidal firing activity following application of 10 μM SKF81297, 5 μM SCH23390 + 50 μM DA, and 10 μM quinpirole.

B. Top panel: Quantitative group analysis of cell excitability, SKF81297 induces a significant decrease in excitability whereas 5 μM SCH23390 + 50 μM DA and 10 μM quinpirole did not have any inhibitory effect. Bottom panel: Input-output plots recorded during each of 4 different treatments (n = 6-10).

C. Left panel: Typical traces illustrating the impact of various DAergic ligands on input resistance. Right panel: 10 μM SKF81297 induces a significant decrease in input resistance whereas 5 μM SCH23390 + 50 μM DA and 10 μM quinpirole did not (n = 6-10).

D. Left panels: Electrophysiological current-clamp traces illustrating 10 μM DHPG-induced persistent firing along with the reversible inhibitory effects of 50 μM DA and 10 μM SKF81297. Right panel: Quantitative group analysis illustrating the significant decrease in firing frequency during persistent firing following application of 50 μM DA or 10 μM SKF81297 in comparison to the control group (DHPG alone) (n = 11-13).

Values represented as means ± SEM: *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

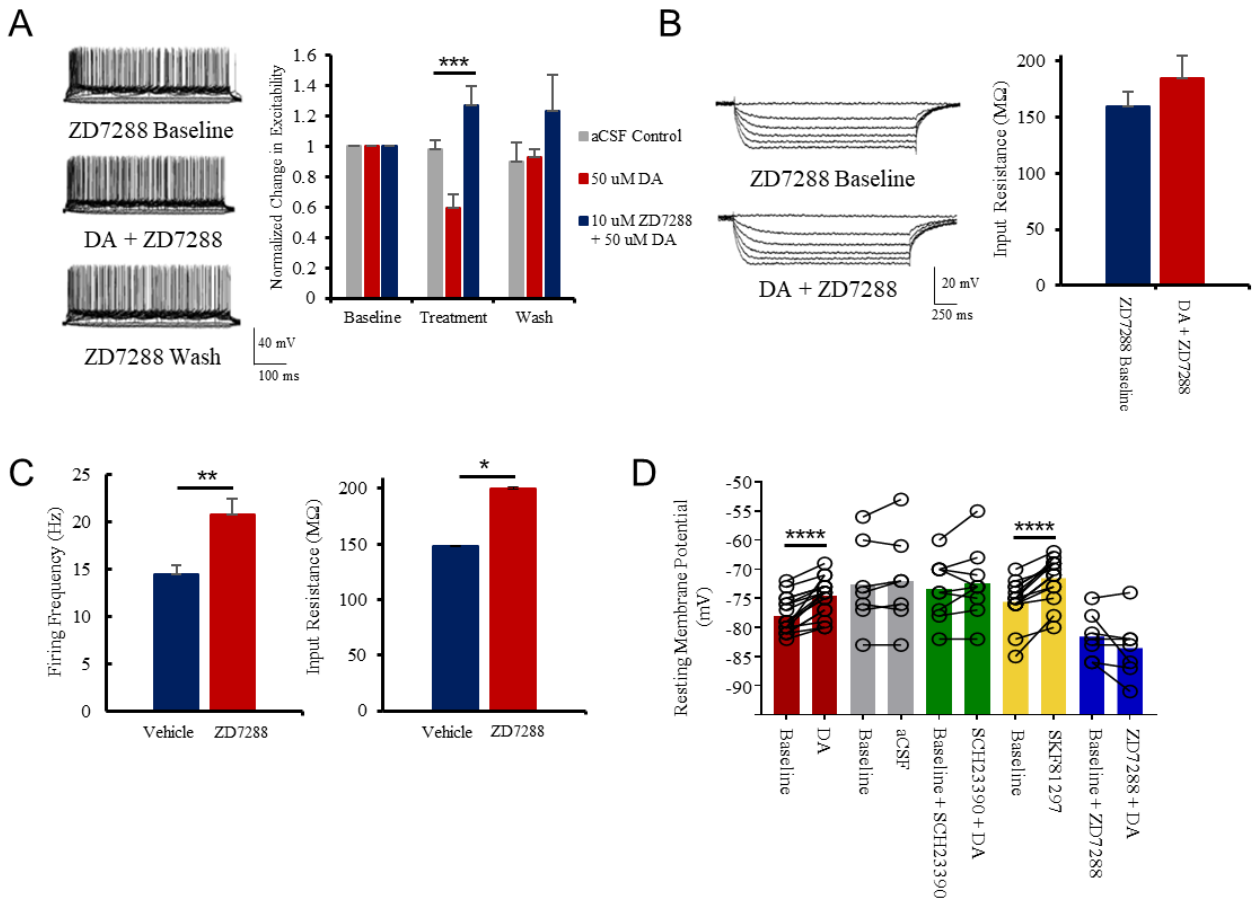

Figure 3. DAergic inhibition depends on HCN channels.

A. Treatment with the HCN channel blocker 10 µM ZD7288 occludes the inhibitory effects of 50 µM DA. Representative current-clamp traces showing the effects of 10 µM ZD7288 on excitability (left) and group analysis (right) (n = 8).

B. 10 µM ZD7288 blocks the 50 µM DA-induced decrease in input resistance. Representative current-clamp traces showing the effects of 10 µM ZD7288 on input resistance (left) and quantitative group analysis (right) (n = 7).

C. Quantitative effects of 10 µM ZD7288 on firing frequency (left) and input resistance (right) at baseline (n = 8).

D. Both 50 μM DA and 10 μM SKF81297 induce a significant increase in resting membrane potentials of layer 2/3 pyramidal neurons of the ACC. This effect is lost in presence of the D1R antagonist SCH23390 (5 μM) or when HCN channels are blocked with ZD7288 (10 μM) (n = 7-17).

Values represented as means ± SEM: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

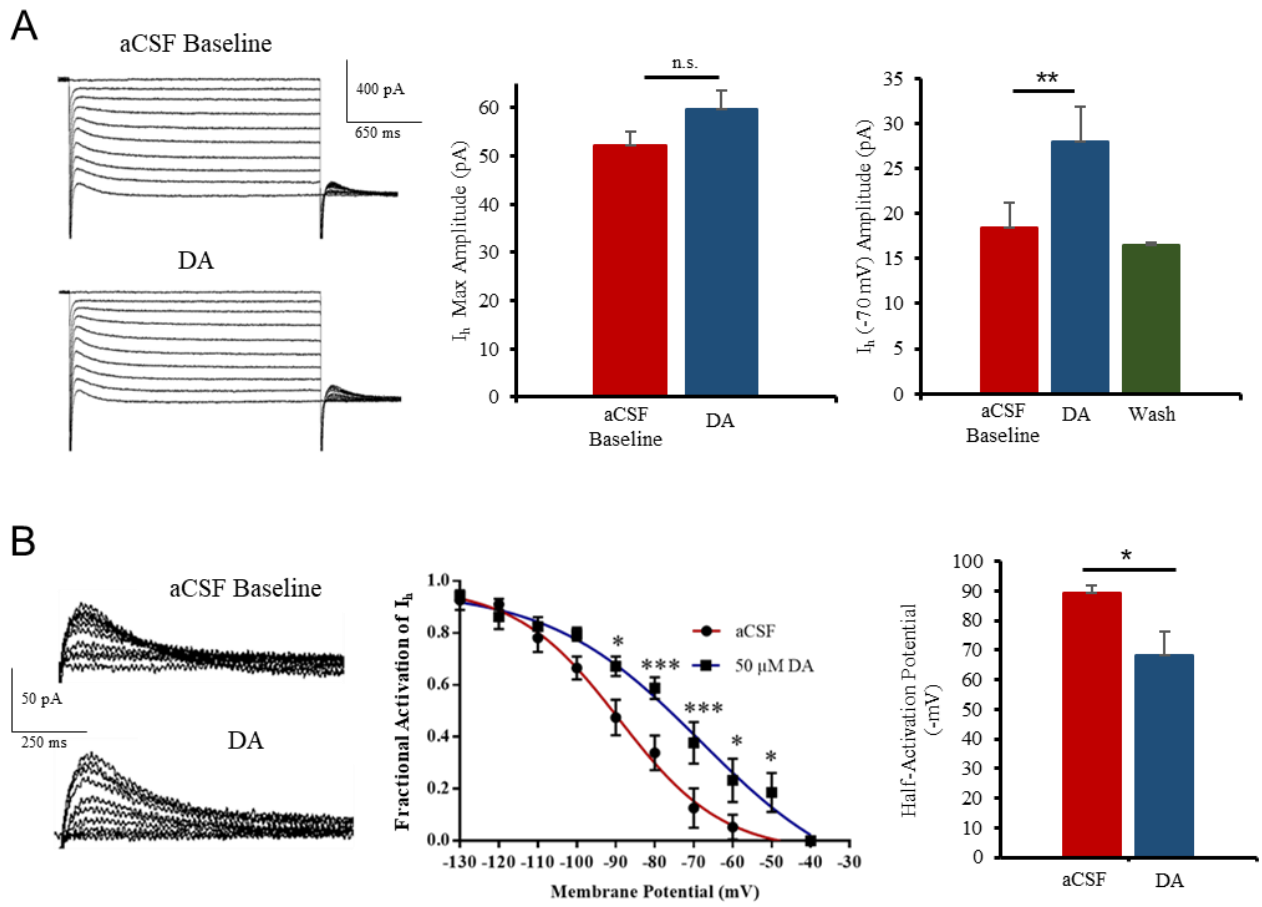

Figure 4. DA regulates HCN channel activity in pyramidal neurons of the ACC.

A. Left panel: Representative voltage-clamp traces showing HCN currents (holding voltage -130 mV to -40 mV, 10 steps) before and after 50 µM DA application in layer 2/3 ACC. Middle panel: No significant increase in max I_h (recorded at -130 mV) following DA application. Right panel: 50 µM DA induces a strong and reversible increase in I_h around physiological membrane potentials (-70 mV) (n = 9).

B. Left panel: Typical tail currents shown. Middle panel: Group analysis fitted with Boltzmann function shows a significant increase in open HCN channels at physiological voltages (-90 mV to

-50 mV). Right panel: Significant depolarizing shift in half-activation potential of HCN channels after 50 μM DA application (n = 5).

Values represented as means ± SEM: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

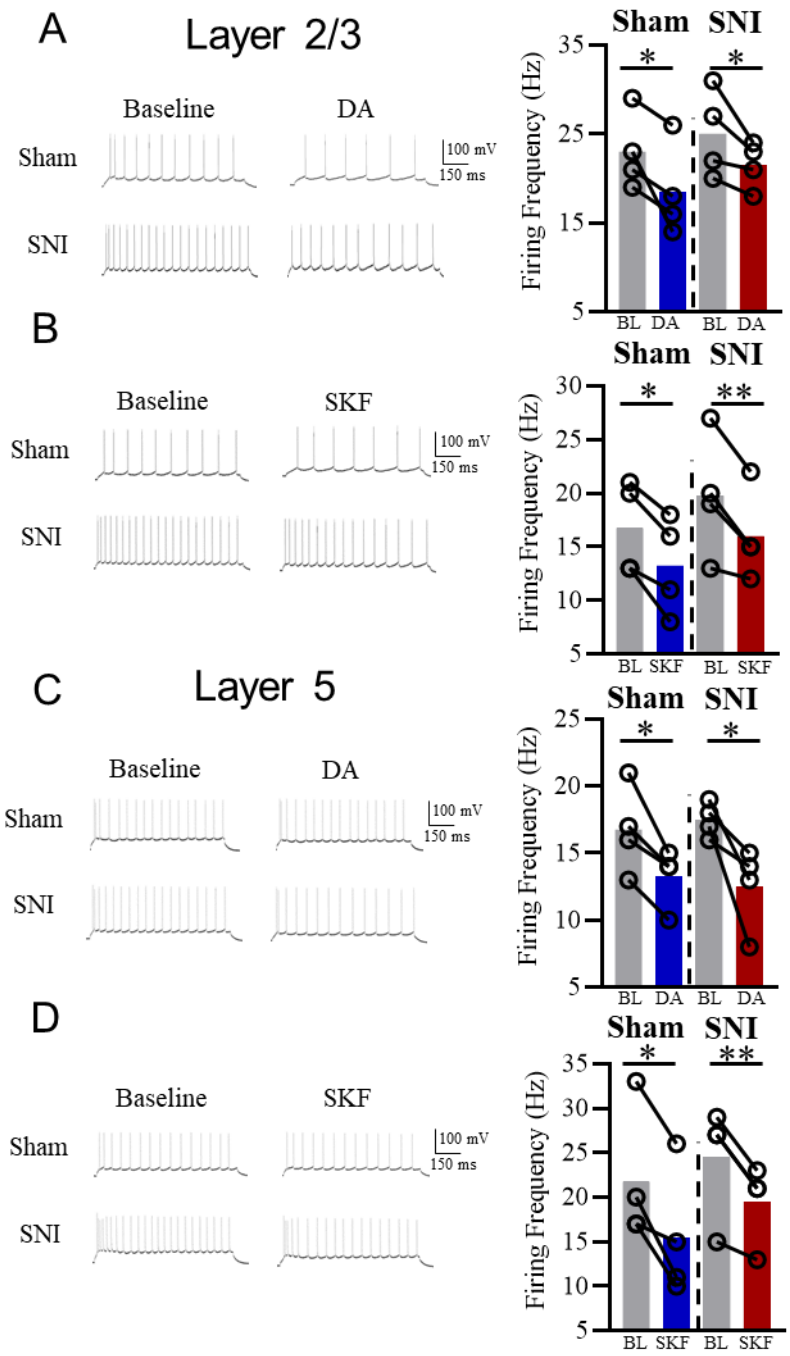

Figure 5. DAergic inhibition preserved in neuropathic mice across cortical layers

A. Typical electrophysiological traces and group analysis illustrating 50 μM DA induces significant inhibition of layer 2/3 ACC pyramidal neurons in sham and SNI mice (+100 pA stimulation) (n = 4).

B. Typical electrophysiological traces and group analysis illustrating 10 μM SKF81297 inhibits layer 2/3 ACC pyramidal neurons in both sham and SNI mice (+100 pA stimulation) (n = 4).

C. Typical electrophysiological traces and group analysis illustrating 50 μM DA induces significant inhibition of layer 5 ACC pyramidal neurons in sham and SNI mice (+100 pA stimulation) (n = 5).

D. Typical electrophysiological traces and group analysis illustrating 10 μM SKF81297 inhibits layer 5 ACC pyramidal neurons in both sham and SNI mice (+100 pA stimulation) (n = 6).

Values represented as means : *$p < 0.05$, **$p < 0.01$.

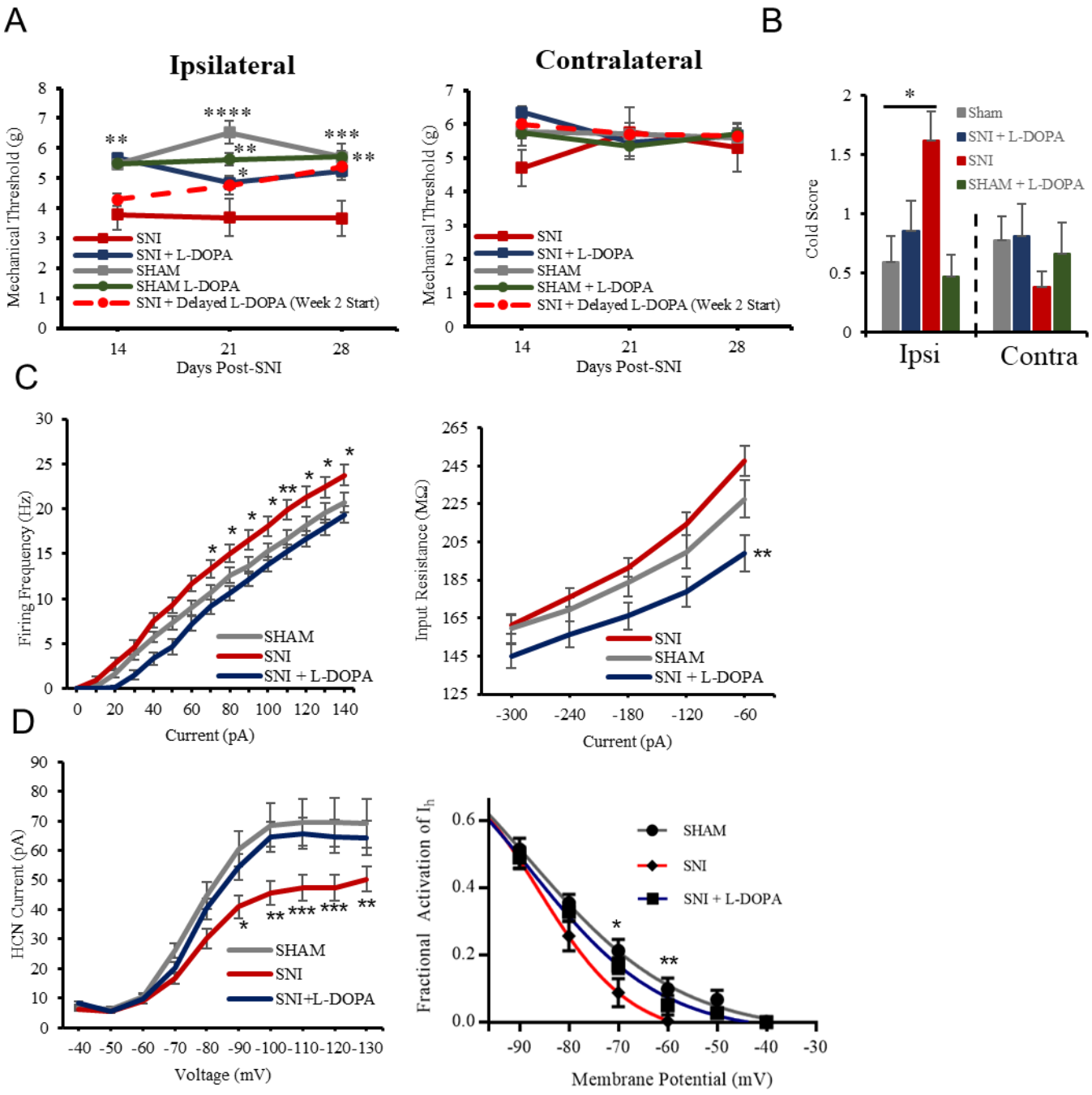

Figure 6. L-DOPA/carbidopa treatment effective at reducing mechanical hypersensitivity in SNI mice.

A. Mechanical withdrawal thresholds on ipsilateral paw (left) and contralateral paw (right) between treatment conditions. Groups: SNI, sham, SNI + L-DOPA, sham + L-DOPA, and SNI +

delayed L-DOPA (supplementation starting two weeks post-SNI surgery rather than on the day of SNI surgery) (n = 6-11).

B. Mean scores of responses to cold in the acetone test on ipsi- and contralateral paws in several treatment conditions (n = 7-11).

C. Input-output plots documenting L-DOPA/carbidopa-induced decrease in both pyramidal excitability (left) and input resistance (right) (n = 13-27).

D. Rescue of HCN channel activity in L-DOPA/carbidopa-treated SNI mice (left), with open channel probabilities on level with sham mice at resting membrane potential (right) (n = 27-34).

Values represented as means ± SEM: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

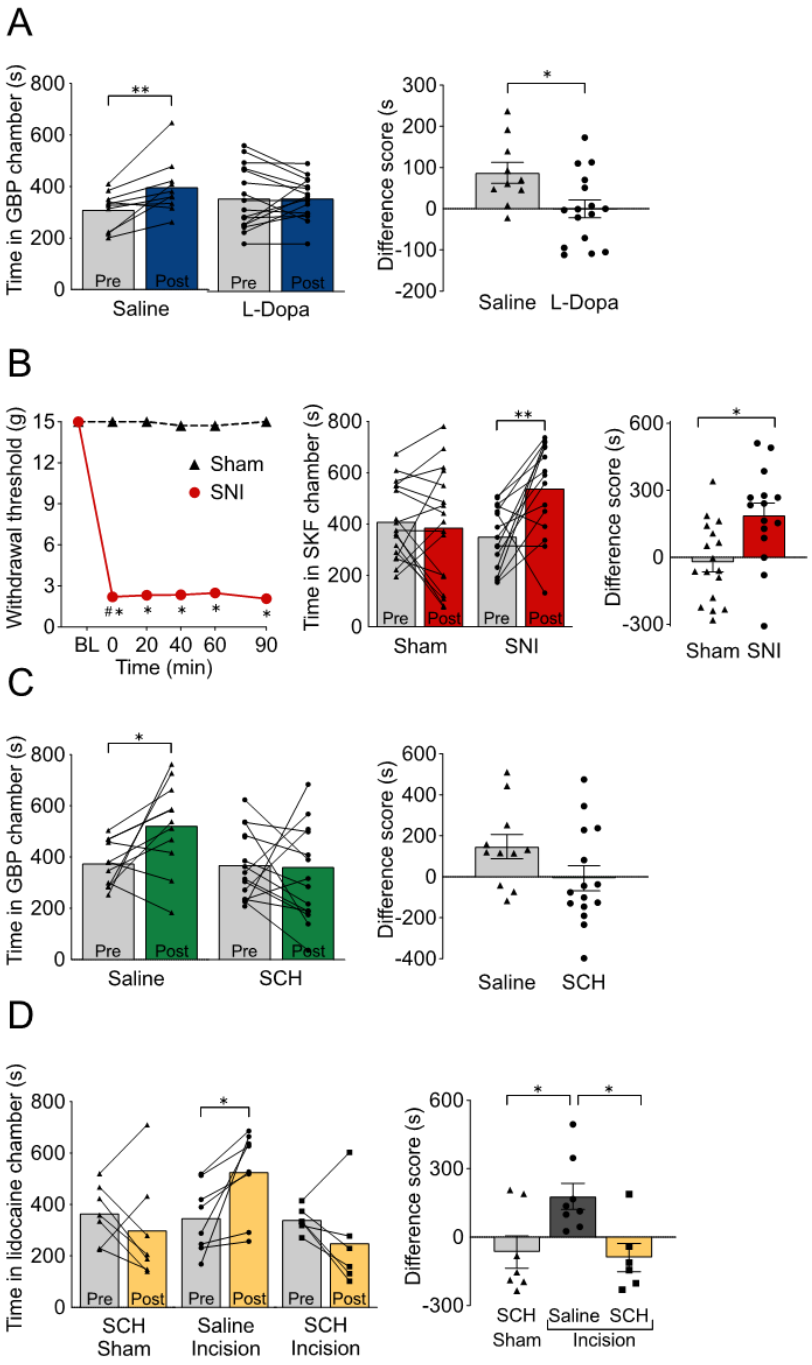

Figure 7. Role of dopamine/D1R signaling in the ACC in pain relief.

A. Systemic L-DOPA/Carbidopa prevents gabapentin-induced CPP in SNI mice. Saline treated SNI mice spent significantly more time in the gabapentin (GBP) paired chamber after

conditioning (Post) compared to the time in the same chamber before conditioning (Pre) . L-Dopa/Carbidopa treated SNI mice did not show significant changes in chamber preference (left). CPP difference scores were calculated as the difference between post-conditioning and pre-conditioning time spent in the GBP-paired chamber (right) (n = 10-16).

B. D1R agonist in ACC produces CPP in SNI rats. Bilateral microinjection of SKF81297 in the ACC (0.5 µg/0.5 µl) had no effect on reflexive tactile responses in either SNI or sham rats. (left). SNI rats spent significantly more time in the SKF81297 paired chamber after drug conditioning (Post) compared to the time in the same chamber before conditioning (Pre)(middle). The difference score for the SKF81297-paired chamber is significantly larger in SNI rats than in sham rats (right) (n = 15-17).

C. D1R antagonist in ACC blocks gabapentin-evoked CPP in SNI rats. Bilateral microinjection of SCH23390 (10 µg/0.5 µl) in the ACC of SNI rats prevents GBP-evoked CPP (left). After drug conditioning (Post), SNI rats microinjected with saline in the ACC spend significantly more time in the GBP-paired chamber than before conditioning (Pre) (left). Difference between post-conditioning and pre-conditioning time spent in the GBP-paired chamber (right) (n = 11-15).

D. D1R antagonist in ACC blocks CPP induced by lidocaine nerve block in a rat model of post-surgical pain. Bilateral microinjection of SCH23390 (10 µg/0.5 µl) in the ACC of rats with left hindpaw incision prevents CPP elicited by peripheral nerve blockade with injection of lidocaine in the popliteal fossa of the injured hindpaw (left). After lidocaine conditioning (Post), injured rats microinjected with saline in the ACC spend significantly more time in the lidocaine-paired chamber than before conditioning (Pre) (left). The difference score for the lidocaine-paired

chamber is significantly larger in saline/incision rats than in SCH/sham or SCH/incision rats (right)(n = 6-8).

Bars represent means ± SEM, symbols show the values for individual animals: *p < 0.0001, **p < 0.01.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SKF81297	Tocris	Cat#1447
Quinpirole	Tocris	Cat#1061
DHPG	Tocris	0805/5
Dopamine	Sigma-Aldrich	Cat#H8502
ZD7288	Tocris	Cat#1000/10
L-DOPA	Sigma-Aldrich	PHR1271
Carbidopa	Sigma-Aldrich	PHR1655
SCH23390	Tocris	Cat#0925
Experimental models: Organisms/strains		
Wild-Type C57Bl6 Mice	Charles River Canada	C57Bl6
Wild-Type C57BL/6J mice	Jackson Laboratories	Stock No: 000664
Sprague-Dawley rats	Harlan Laboratories	#002
Software and algorithms		
pClamp	Molecular Devices	RRID:SCR_011323
Prism 9	Graphpad	RRID:SCR_002798
ANY-maze	ANY-maze	RRID:SCR_014289

STAR Methods

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Philippe Séguéla (philippe.seguela@mcgill.ca).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

1. All data reported in this paper will be shared by the lead contact upon request.
2. This study did not generate code.
3. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Mice Subject Details

Six-week old male C57BL/6 mice (Charles River Laboratories, QC, CA) were housed in the Montreal Neurological Institute (MNI) Animal Care Facility (ACF) and all procedures are following the Canadian Council on Animal Care guidelines. For CPP experiments, six-week old male C57BL/6J mice (Jackson Laboratories) were housed in the University of Arizona ACF. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona and were in accordance with guidelines from the International Association

for the Study of Pain. Mice were kept in a 12 hour light-dark cycle with free access to water and food.

Rat Subject Details

Eight to nine week old male Sprague-Dawley rats, 250-300g (Harlan Laboratories, Indianapolis, IN), were housed in the University of Arizona ACF. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona and were in accordance with guidelines from the International Association for the Study of Pain. Rats were kept in a 12 hour light-dark cycle with free access to water and food.

Method Details

Spared Nerve Injury (SNI) and Incisional Injury Models

Isoflurane (2%) and ketamine/xylazine (80/12 mg/kg i.p.) were used to anesthetize mice and rats, respectively. Spared nerve injury (SNI) surgery to induce chronic neuropathic pain was performed on mice and rats 14 days prior to CPP and von Frey testing. For SNI, the tibial and peroneal nerves were ligated and cut, leaving the sural nerve intact. Incisional injury was performed according to the Brennan model (Brennan TJ, Vandermeulen EP, & Gebhart GF (1996) Characterization of a rat model of incisional pain. Pain 64(3):493-501) as described previously (Navratilova, Proc Natl Acad Sci U S A. 2012 Dec 11;109(50):20709-13).

Electrophysiological Recordings

Animals were anesthetized with an Avertin solution (2.5 g tribromoethanol in 5 mL amylene hydrate diluted in 100 mL $_{dd}H_2O$) (Sigma-Aldrich, St. Louis, MI). Then animals were transcardically perfused with a 4^{o}C choline-chloride based cutting solution oxygenated with

carbogen (O_2 95%, CO_2 5%) (Praxair). Brains were extracted and sliced into 300 µm sections using a vibratome (Leica VT1000). Slices were allowed to rest at room temperature for 1 hour in oxygenated (see above) artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 2 mM KCl, 26 mM $NaHCO_3$, 1.8 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 10 mM Glucose, 1.6 mM $CaCl_2$, pH 7.4. Slices were submerged at 30-32^oC on the stage of a Zeiss Axioskop microscope continuously perfused with oxygenated (see above) aCSF containing 1.8 mM Kynurenic Acid and 100 µM picrotoxin at a rate of 1 mL/minute. A near-infrared CCD camera coupled to a x63 water immersion objective was used during patch-clamp recordings (Sony XC-75). Cells were patched with BF150-75-10 glass pipettes (~ 6 MΩ) pulled with a Flaming Brown Micropipette Puller (Model P-97, Sutter Instruments, US). Pipettes were mounted on a MP-225 micromanipulator and filled with an intracellular solution containing 120mM K-gluconate, 10 mM HEPES, 0.2 mM EGTA, 20 mM KCl, 2 mM $MgCl_2$, 7 mM diTrisPhosphate-Creatine, 4 mM Na_2ATP, and 0.3 mM NaGTP (Sutter Instruments, Novato, CA). An Axopatch 200B amplifier and Digidata 1322A interface digitizer were used for data acquisition (Molecular Devices, San Jose, CA). Signals were low-pass filtered at 10 kHz for current-clamp recordings and at 2 kHz for voltage-clamp recordings, digitized at 20 kHz.

Neurons were patched in areas corresponding to layer 2/3 and layer 5 dACC according to stereotaxic coordinates (Paxinos et al, 2008). Pyramidal cells were identified based on firing frequency, input resistance (MΩ), and spike adaptation to a 1-second 100 pA cuurent injection pulse. Cells with resting membrane potentials (RMP) above -50 mV or below -80 mV were excluded. Series resistance was compensated (≤ 35 MΩ).

Excitability was evaluated by bringing cells to -60 mV RMP and counting action potentials evoked by a 17-step protocol (current input ranging from -20 pA to +140 pA, 10 pA steps). Input-output

was calculated based on action potentials evoked at each step. Input resistance (MΩ) was assessed by injecting cells with a 6-step protocol (current input ranging from -300 pA to 0 pA, 60 pA steps). Input resistance was averaged across steps and evaluated across treatments.

HCN induced *sags* were measured in layer 5 pyramidal neurons using whole-cell patch recording in current-clamp mode; two current steps of -300 pA and -120 pA were used to record max *sag* and *sag* at physiological relevant potentials, respectfully. *sag* ratios were calculated by measuring the *sag* amplitude and normalizing it to the steady-state voltage at the end of each current step, shown below.

$$\text{SAG Ratio} = \frac{V_{Baseline} - V_{sag\ Minimum}}{V_{Baseline} - V_{Steady-state}}$$

HCN currents (I_h) were measured in voltage clamp mode in the additional presence of 1 µM TTX and 200 µM $BaCl_2$ to block voltage-gated sodium channels and inward rectifying potassium channels (K_{ir}), respectively. Cells were held at a holding potential of -40 mV followed by a 2.5 second step ranging from -40 mV to -130 mV holding voltage (10 step protocol, 10 mV steps). Following each respective step, cells were held at -130mV for 1 second to record tail currents. Current amplitudes of HCN channels were measured by calculating the difference between the current at the start of each step (during the peak) and at the end of each step during the plateau phase. Physiological I_h was measured at -70 mV and max I_h was determined at -130 mV holding voltage. Fractional activation of I_h was calculated by measuring the amplitude of the tail current after each step and normalized to the max tail current (see equation below). A Boltzmann function was fitted using GraphPad Prism 7 (GraphPad Software).

$$\text{Fractional Activation of } I_h = 1 - \frac{I_{tail}(V)}{I_{tail}(max)}$$

I_h amplitude was assessed by obtaining baseline values 5 minutes after patching and comparing them with values obtained following 10-minute treatment with 50 μM DA. Resting membrane potential (mV), temperature, series resistance (MΩ), and whole-cell capacitance were measured before each time point.

Intracranial ACC Cannulation

Stereotaxic cannulation surgeries were performed in animals anesthetized with a ketamine/xylazine combination (80/12 mg/kg, i.p.; Western Medical Supply/Sigma, Arcadia, CA). Bilateral cannulation of the ACC was performed as previously described (Johansen et al, 2004; Navratilova et al, 2015). A pair of 26-guage stainless steel guide cannulas cut 4 mm below the pedestal (Plastics One Inc., Roanoke, VA) were directed toward the following ACC injection site: AP: +2.6 mm from bregma; ML: ± 0.6 mm; DV: -1.8 mm from skull). Guide cannulas were cemented in place and secured to the skull by small stainless-steel machine screws. Stainless steel dummy cannulas were inserted into each guide to keep the guide free of debris. Rats then received a subcutaneous gentamycin (1 mg/ml) injection, were housed individually, and were allowed to recover for 7-10 days. Cannula locations were verified after the experiment; 10 rats were eliminated due to incorrect placement.

L-DOPA/Carbidopa Dosage and Administration for Electrophysiology and von Frey Assay

Mice were placed on a strict L-DOPA/Carbidopa regimen immediately following SNI surgery. Light-shielded L-DOPA and carbidopa were dissolved in their water supply and administered continually throughout the duration of the study (Blunt et al, 1993). Water intake per mouse was measured 3 times weekly and L-DOPA/carbidopa concentrations were adjusted so as each mouse received 75 mg/24 hr/kg L-DOPA and 10 mg/24 hr/kg carbidopa. In the case of delayed L-DOPA

treatment group, administration of L-DOPA/carbidopa started after the first day of behavioral testing instead of immediately following SNI surgery.

Behavioral Tests

Von Frey: Animals were placed in suspended chambers with wire mesh floors for 30 minutes for habituation prior to testing. Mouse withdrawal thresholds were determined by stimulating the lateral planar surface of both the ipsi- and contralateral paws using an automatic von Frey apparatus (Ugo Basile, Italy). Rat withdrawal thresholds were calculated using a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL). Logarithmically spaced increments ranging from 0.41 to 15 g (4–150 N) were applied perpendicular to the plantar surface of the ipsilateral hind paw until the filament buckled. Withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength ("up and down" method), analyzed using a Dixon nonparametric test, and expressed as the mean withdrawal threshold (Dixon 1980).

Acetone cold test: Cold allodynia was measured by applying a drop of acetone to the lateral planar surface of the paw and measuring acute behavioral responses of the mice (0 = no response, 1 = hind paw flinching and stamping, 2 = hind paw licking, 3 = exaggerated response and/or vocalization).

Conditioned place preference (CPP):

Mice: SNI mice were pretreated daily with either saline or L-DOPA/carbidopa (20 mg/10ml/kg/ 24hr and 4 mg/10ml/kg/ 24 hr, i.p.) for 12 days starting on the day of SNI surgery. Mice were acclimated to CPP chambers on day 11 after surgery and baselines were assessed on day 12. Conditioning was performed on days 13 and 14 with morning injections of saline and confinement for 30 min in one chamber and gabapentin (30 mg/ml/kg, i.p.) injections followed by 30 min

confinement in the opposite chamber in the afternoon. On test day (day 15), mice were placed in the middle chamber with free access to both conditioning chambers and the time spent in each chamber was recorded for 15 min. Ten mice were eliminated due to failed surgery or failed baselines.

Rats: A single trial conditioning protocol was used for CPP as previously described (King et al, 2009; Navratilova et al, 2013). On preconditioning day, rats were placed into the CPP boxes with free access to all three chambers. Anymaze software was used to determine the time spent in each chamber across 15 min. Animals spending more than 80% (720 s) or less than 20% (180 s) of the total time in either chamber were eliminated from further testing (19 rats).

Conditioning day - microinjection of SFK81297: SNI and sham rats with ACC cannulas received a saline injection into the ACC and were placed into a conditioning chamber for 30 min. Four hours later, rats received SKF81297 (0.5 µg/0.5 µl) into the ACC and were placed into the opposite chamber for 30 min.

Conditioning day- microinjection of SCH23390: SNI rats with ACC cannulas were pretreated with bilateral microinjections of either saline (0.5 µl) or SCH23390 (10 µg/0.5 µl) in the ACC. Following pretreatment, rats received a saline injection (i.p.) and placed into a conditioning chamber for 30 minutes. Four hours later, pretreated SNI rats received gabapentin (50 mg/ml/kg, i.p.) and placed into the opposite chamber for 30 minutes. The experiment with incision rats was performed in a similar way but in the afternoon, rats received injection of lidocaine (200 µl; 4%) into popliteal fossa under light isoflurane anesthesia to induce peripheral nerve blockade.

On test day, rodents were placed drug free into the middle CPP chamber and were allowed to explore all chambers for 15 minutes; the time in chambers was assessed using the Anymaze

software to determine chamber preference. Difference scores were calculated as test time minus preconditioning time spent in the drug-paired chamber.

Drugs and Solutions

Reagents used for aCSF, cutting solution, internal pipette solution, DA, carbidopa, and xylazine were purchased from Sigma-Aldrich (St. Louis, Missouri). TTX, DHPG, SKF81297, ZD7288, quinpirole, and SCH23390 were obtained from Tocris (Bristol, UK). Levodopa was purchased from Santa Cruz Biotechnology (Dallas, Texas). Ketamine was obtained from Western Medical Supply (Arcadia, CA). All drugs were diluted and aliquoted into single-use samples stored at -20^{o}C. Gabapentin was purchased from Spectrum Chemical MFG (Gardena, CA) and prepared fresh by dissolving in distilled water. Lidocaine was purchased from Qualitest Pharmaceuticals.

Quantification and Statistical Analysis

All data were analyzed using Clampfit 10 and statistical analysis was performed using GraphPad Prism 7. Values are represented as either raw or normalized means ± SEM with significance threshold set at $p < 0.05$. One-way ANOVA with Dunnett's multiple comparison was used to compare multiple independent groups (aCSF vs 10 µM DA vs 50 µM DA). Unpaired t-tests were used to compare two independent groups whereas the paired t-test was used to compare two related groups (baseline vs treatment). Two-way ANOVA with Bonferroni multiple comparison was used to analyze significance between independent samples under multiple conditions. Significance for fractional activation of HCN channels was tested using repeated measure ANOVA. CPP chamber preference was analyzed using the Anymaze software (Wood Dale, IL).

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Linker

Chapter 2 provides conclusive evidence that DA is a potent neuromodulator of pyramidal cells in L2/3 and L5 of the ACC. Activation of post-synaptic D1R mediates the opening of HCN channels at relevant membrane potentials, decreasing input resistance and consequently excitability. Furthermore, we show intracortical injection of D1R agonists in the ACC is analgesic. Given the diverse physiological roles of the ACC, this proves that dopaminoceptive pyramidal cells expressing D1R are involved in top-down pain modulation.

Moreover, we demonstrate that supplementing supraspinal DA in neuropathic rodents alleviates symptoms of chronic pain and occludes pathological ACC hyperexcitability. This is mediated by correcting the chronic pain-induced HCN dysfunction present in pyramidal neurons of the ACC. Neuropathic animals supplemented with L-DOPA and carbidopa displayed increased open channel probabilities of HCN channels at physiological membrane potentials, decreasing input resistance and excitability. Moreover, we present evidence that the pain relief provided by L-DOPA occludes the pain relief provided by gabapentin, a commonly prescribed analgesic in chronic pain patients, and D1R signaling in the ACC is required for effective pain relief. These results irrefutably prove that mesocortical DA signaling in the ACC is fundamentally integrated in top-down pain modulation and decreased DAergic input appears to promote pathological hyperexcitability in neuropathic conditions.

Given this, it is critical to understand which experiences impact DA release in the ACC and whether they are affected by the onset of chronic pain. Reward and pain have been shown to modulate the activity of DAergic cells in the VTA but how that translates into changes in DA release in the ACC is not established. Furthermore, DA receptors are expressed on inhibitory

interneurons in the ACC and the role of DA in mediating inhibitory inputs onto pyramidal cells is poorly understood. If DA is shown to affect GABA-mediated inhibition of pyramidal neurons, it could play a converging role in chronic pain-induced disinhibition in the ACC.

In conclusion, Chapter 3 will take advantage of the recent advances in GENIs to give us an unparalleled opportunity to interrogate which behaviors are involved in mediating DA release in the ACC and whether neuropathic pain affects them. Additionally, we will interrogate the role of DA and the mesocotical pathway in GABAergic inhibition of pyramidal neurons and determine if this pathway is affected in chronic pain states.

Chapter 3

Chronic Pain Alters Dopamine Release in Anterior Cingulate Cortex

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Summary

Dopamine (DA) has been shown to be a potent neuromodulator of the anterior cingulate cortex (ACC), a prefrontal region involved in processing the affective components of pain that displays hyperexcitability in chronic pain. However, which experiences influence DA release in the ACC and whether they are affected by chronic pain has yet to be investigated. We show here a novel D1R-mediated GABA-dependent inhibitory mechanism which reduces ACC pyramidal firing frequency by increasing spontaneous inhibitory post synaptic currents (sIPSCs). We also demonstrate, using *in vivo* photometry in freely-moving mice, that reward and pain bidirectionally control DA release in the ACC, increasing or decreasing DA, respectively. Using different pain modalities, we show that DA release in ACC is inhibited proportionally to the intensity of the noxious stimuli. Furthermore, pain-induced inhibition of DA release is potentiated following pain chronification, resulting in a prolonged hypodopaminergic state. These results are critical to understanding dysregulations in cortical circuits involved in top-down pain modulation.

Keywords: dopamine, D1 receptor, inhibitory post synaptic currents, photometry, chronic pain, top-down pain modulation, acute pain, neuromodulation, monoamines

Introduction

The cause of chronic pain-induced pyramidal hyperexcitability in the anterior cingulate cortex (ACC), a region of the prefrontal cortex heavily implicated in affective components of pain, is poorly understood (Cordeiro Matos et al, 2015; Eto et al, 2011; Kasanetz et al, 2022; Kuner et al, 2017; Lançon et al, 2021; Mhalla et al, 2010; Rainville et al, 1997). Decreasing ACC hyperexcitability has been shown to alleviate symptoms of chronic pain, notably hyperalgesia and allodynia, in several rodent models (Gu et al, 2015; Kang et al, 2015; Sellmeijer et al, 2018). Thus, understanding the dysregulated cortical circuitry present in neuropathic conditions could be critical to the development of novel analgesic treatments. Recent studies have identified several key neuromodulators of the ACC, such as serotonin, norepinephrine, dopamine, cannabinoids, orexin, oxytocin, and opioids (Cordeiro Matos et al, 2018; Esmaeilou et al, 2022; Hoot et al, 2010; Karimi et al, 2019; Lançon et al, 2021; Navratilova et al, 2015; Santello et al, 2017). However, it remains unknown how chronic pain affects these pathways and whether they are involved in promoting cortical hyperexcitability.

The strong comorbidity between chronic pain and hypodopaminergic disorders makes DA a prime suspect in promoting cortical hyperexcitability (Blanchet et al, 2018; Defazio et al, 2008; Djaldetti et al, 2004; Ford 2010). Non-myogenic chronic pain is highly prevalent in Parkinsonian patients while supplementing supraspinal DA via L-DOPA attenuates pain symptoms and decreases ACC activity (Brefel-Courbon et al, 2005). We have previously reported that DA is a potent inhibitory neuromodulator of ACC pyramidal excitability via a D1R-mediated HCN- dependent mechanism. We have also shown that systemic L-DOPA decreases hyperalgesia and normalizes ACC hyperexcitability in mice with chronic pain (Lançon et al, 2021). Moreover, a recent clinical study demonstrated that small doses of L-DOPA can alleviate chronic back pain in humans (Reckziegel

et al, 2019). Coupled to these findings, the fact that the ventral tegmental area (VTA), a primary source of DA in the ACC , is pathologically hypoactive in chronic pain, suggests that a deficit in DAergic signaling in the ACC in chronic pain conditions contributes to the development of cortical hyperexcitability (Huang et al, 2019; Ko et al, 2018). Given this, it is critical to understand which experiences influence DA release in the ACC and how the onset of chronic pain impacts them.

Pain has been shown to decrease DA release in the midbrain. Recent studies have identified a peripheral pathway activated by nociception responsible for inhibiting VTA neurons and demonstrated that capsaicin-evoked tonic pain significantly decreases DA release in the nucleus accumbens (Gee et al, 2020; Yang et al, 2021). Conversely, rewarding stimuli activate the mesolimbic pathway and provide pain relief (Hernandz and Hoebel, 1988; Atlas and Wagner, 2012). Similarly, pain relief promotes DA release and D1R signaling in the ACC and we have shown it is necessary for effective pain relief in neuropathic and post-surgical pain models (Lançon et al, 2021). Yet, how other behavioral responses, such as reward and pain, affect DA release specifically in the ACC remains unclear and warrants investigation.

In the present study, we used *in vivo* photometry to investigate cortical DA release following reward and three types of pain modalities: cold, chemical, and acute pain. We found that reward and pain bidirectionally control the release of DA in the ACC. Rewarding stimuli promotes whereas pain inhibits the release of DA in the ACC. Furthermore, we provide evidence that the onset of chronic pain significantly increases ACC pyramidal calcium events and potentiates noxious stimuli-induced inhibition of DA release. We also demonstrate a novel inhibitory mechanism in the ACC reliant on D1R-induced GABA release. Although we have shown that the HCN-mediated D1R-dependent inhibitory pathway is preserved in chronic pain, this GABAergic pathway loses some functionality (Lançon et al, 2021). These results are a step forward in

understanding how longitudinal alterations in neurotransmitter release in the ACC promote pathological pyramidal neuron hyperexcitability and mediates hypersensitivity in chronic pain conditions.

Results

ACC D1R Signaling Inhibits Pyramidal Cells via GABA Signaling

We have previously reported that D1R activation in ACC layer 2/3 significantly inhibits roughly half of synaptically-isolated pyramidal neurons (Lançon et al, 2021). To determine if GABA signaling also plays a role in D1R-mediated inhibition of pyramidal neurons in layer 2/3 ACC, we applied 10 µM SKF81297 in the absence of the $GABA_A$ antagonist picrotoxin. Following 10 minute bath application of 10 µM SKF81297, 100% of layer 2/3 pyramidal neurons were inhibited relative to baseline (n = 9 of 9)(Figure 1A). The firing frequency of inhibited cells, evoked by a 500 ms +100 pA pulse, was decreased from 12.0 ± 2.1 Hz to 9.5 ± 1.7 Hz following 10 µM SKF81297 application (n = 9, paired t-test: $t(8) = 4.6$, $p = 0.0017$) (Figure 1A). Following a 10-minute washout, 88.8% of cells displayed a firing frequency not significantly different from baseline values (n = 8, paired t-test: $t(7) = 1.5$, $p = 0.1735$)(Figure 1A).

D1R Activation Increases sIPSCs on ACC Pyramidal Cells

To determine why removing picrotoxin had such a significant effect on the percentage of cells inhibited by D1R activation, we investigated SKF81297-induced inhibitory post-synaptic currents in the presence (mIPSCs) and absence (sIPSCs) of the voltage-gated sodium channel blocker TTX. Our results demonstrate that 10 µM SKF81297 drastically increased the frequency of sIPSCs but was without significant effect on mISPCs, indicating that presynaptic action potentials are required for D1R-evoked GABA release (Figure 1B). The frequency of sIPSCs significantly increased from 2.4 ± 0.6 Hz to 4.1 ± 0.5 Hz, and again to 5.2 ± 0.7 Hz following 5 and 10 minute application of 10 µM SKF81297, respectively (n = 6, paired t-test, 5 minute: $t(5) = 3.082$, $p = 0.27$; 10 minute: $t(5) = 2.8$, $p = 0.036$)(Figure 1C). Interestingly, although at first glance it appears the amplitude of

sIPSCs increases following D1R activation, it was not found to be significant (from 33.0 ± 3.15 pA to 61.53 ± 18.1 pA, Figure 1C). This is further demonstrated by the cumulative probability plots, which clearly illustrate the decrease in intervals between sIPSC events but not the amplitude (Figure 1D).

Unlike sIPSCs, 10 µM SKF81297 had no effect on the frequency or the amplitude of mIPSCs (Figure 1C). Following application of 10 µM SKF81297, the frequency of mIPSCs remained unchanged, from 3.30 ± 0.72 Hz to 3.02 ± 0.70 Hz (Figure 1C). Similarly, the amplitude of mIPSCs is unaffected by SKF81297 (from 19.36 ± 2.07 pA to 18.80 ± 3.10 pA, Figure 1C).

These electrophysiology results suggest D1R activation in the ACC promotes the release of GABA from inhibitory interneurons, facilitating a reversible inhibition of pyramidal cell firing frequency.

GABA-Mediated Inhibition of ACC Pyramidal Cells is Decreased in Chronic Pain

Our group has previously demonstrated that SNI had no significant impact on the amplitude of inhibition or the percentage of inhibited pyramidal cells in the ACC through D1R-induced HCN-mediated inhibition (Lançon et al, 2021). To determine if this is also the case for GABA-mediated inhibition, we tested the effect of 10 µM SKF81297 on the firing frequency of layer II/III pyramidal cells in mice 3 weeks following SNI. In SNI conditions, only 25.0% of cells were inhibited relative to baseline following application of SKF82197 (n = 3 of 12) (Figure 2A). Given these results, we next tested if SKF81297 increased the frequency or amplitude of sIPSCs in layer II/III pyramidal cells in SNI conditions. In concordance with a lack of D1R-mediated GABA-dependent inhibition of ACC pyramidal neurons in SNI mice, we confirmed that SKF81297 had no effect on sIPSCs. Interestingly, in SNI mice, 5 and 10 minute application of 10 µM SKF81297 had no significant impact on the frequency (from 3.41± 0.82 Hz to 3.92 ± 1.03 Hz; n = 6, paired

t-test: t(5) = 0.36, p = 0.73) or amplitude of sIPSCs (from 39.21 ± 8.07 pA to 48.72 ± 9.83 pA; n = 6, paired t-test: t(5) = 0.67, p = 0.5399)(Figure 2B and 2C). Comparing D1R-induced sIPSCs in sham and SNI mice clearly shows the decrease of GABA-mediated inhibition in neuropathic mice, and explains why D1R activation does not effectively inhibit pyramidal cells in neuropathic ACC (Figure 2D). Analysis of inter-event intervals of sIPSCs following application of SKF81297 further illustrates this discrepancy between sham and SNI mice (Figure 2F).

We then investigated whether the baseline frequency or amplitude of sIPSCs in ACC layer II/III pyramidal cells is affected by chronic pain. Our results indicate that chronic pain had no significant effect on frequency or amplitude of sIPSCs in the absence of SKF81297 (frequency: n = 9, unpaired t-test: t(8) = 0.42, p = 0.69; amplitude: n = 9; unpaired t-test: t(8) = 0.96, p = 0.37) (Figure 2E).

Conditioned Reward Increases DA Release in the ACC

Rewarding stimuli are known to activate VTA DAergic neurons and to increase DA release in the mesolimbic pathway (Hernandz and Hoebel, 1988). However, it remains unknown if rewarding stimuli also increase DA release in the mesocortical pathway, specifically in the ACC. To determine if rewarding stimuli can regulate cortical DA release, we used *in vivo* photometry of freely-moving mice expressing the DA sensor dLight1.1 in the ACC (Figure 3A). Mice were trained to press a sporadically available lever for a 15% sucrose solution reward over the course of one week. Our results reveal that both lever presses (data not shown) and nose-pokes (Figure 2B), indicating when the mouse receives the reward, evoked significant release of DA in the ACC. Analysis of dLight1.1 dF/F signals shows a phasic increase of DA release in the ACC, from 0.01± 0.09 dF/F at baseline to 3.47 ± 0.76 dF/F following sucrose reward (n = 4 mice, 40-95 trials per

mouse; paired t-test: t(3) = 5.00, p = 0.0154)(Figures 2B and 2C). This reward-evoked release of DA in the ACC is short-lived, with a return to baseline 5 seconds after the mice receives the reward.

Tonic Pain, but not Acute, Decreases ACC DA Release in Naïve Mice

To test whether painful stimuli also decrease DA release in the ACC, we injected 1% capsaicin intradermally (i.d.) in the feet of mice expressing dLight1.1 in the ACC. Our results demonstrate that capsaicin-induced pain significantly reduces the release of DA in the ACC for 6 to 8 minutes (Figure 3B). dLight1.1 fluorescence drops from 0.18 ± 0.09 dF/F to -4.12 ± 0.85 dF/F 2 minutes following capsaicin injection (n = 3 mice, 1-3 trials per mouse; paired t-test: t(2) = 4.25, p = 0.0256)(Figure 2B and 2C).

Since capsaicin-induced pain lasts for several minutes, we investigated the effects of different pain modalities on ACC DA release. To evoke acute pain, we used 300 ms pulses of 7.5 – 8.5 mW 473 nm blue light directed at the feet of $Na_v1.8^{ChR2\text{-}EGFP}$ mice. This acute stimulation of peripheral nociceptive fibers expressing the sodium channel $Na_v1.8$ causes a rapid, short term pain response in mice. Using dual color photometry, with red-shifted CAMKII promoter-driven calcium sensor jRCaMP1b and dLight1.1 (Figure 4A), we can observe pyramidal cell calcium events and DA release simultaneously in the ACC following "opto-pain" stimulation. We observed that stimulation of $Na_v1.8$+ fibers causes a significant time-locked increase in pyramidal calcium events or activity (Figure 4D). jRCaMP1b fluorescence increases from -0.016 ± 0.035 dF/F to 1.39 ± 0.63 dF/F 3 seconds after stimulation (n = 28, paired t-test: t(27) = 2.227, p = 0.035)(Figure 4D). The effect of pain stimulation on calcium events is better illustrated when analyzing spontaneous calcium events before and after stimulus. The frequency of calcium events, analyzed during the 60 seconds before and after stimulation, increased from 0.03 ± 0.01 Hz to 0.09 ± 0.02 Hz (paired t-test: t(11) = 3.55, p = 0.005)(Figure 4F). The amplitude of spontaneous calcium

events was 2.7 ± 0.15 dF/F z-score (Figure 4E). Surprisingly, we notice no significant effect on dLight1.1 fluorescence following this type of pain stimulation (Figure 4B and 4H). Following stimulation of $Na_v1.8$+ nociceptive fibers, dLight1.1 dF/F, i.e. the relative level of extracellular DA, decreased from -0.02 ± 0.05 to -0.42 ± 0.16 (Figure 4H).

Next, we investigated the effect of cold pain, a highly aversive sensory modality, on DA release and calcium events in the ACC (Colburn et al, 2007). Cold pain was induced by application of 30 µL acetone on the plantar surface of the mouse foot and nocifensive behaviors lasted between 10 and 30 s (Supplementary Video). Our results indicate acetone-evoked cold pain produces a robust calcium response in ACC pyramidal neurons and significantly reduces DA release. Following acetone pain stimulation, the frequency of spontaneous pyramidal calcium events significantly increased from 0.03 ± 0.01 Hz to 0.16 ± 0.02 Hz (n = 17, paired t-test: $t(16) = 7.38$, $p < 0.0001$) (Figure 5D). The amplitude of calcium events was 2.57 ± 0.13 dF/F z-score (Figure 5C). Unlike $Na_v1.8$ stimulation, we noticed dLight1.1 fluorescence significantly decreases following acetone application (Figure 5B, 5E, and 5F). dLight1.1 fluorescence decreases from 0.006 ± 0.19 dF/F to -1.81 ± 0.21 dF/F 3 seconds after cold pain stimulation (n = 18, paired t-test: $t(17) = 4.3$, $p = 0.0005$)(Figure 5B and 5E). This decrease in dLight1.1 fluorescence was short lived, lasting roughly 30 seconds until full return to baseline (Figure 5F).

SNI Increases Calcium Events in ACC Pyramidal Neurons

As chronic pain is known to cause pyramidal hyperexcitability in the ACC, we investigated pain-evoked calcium events in mice before and after a peripheral nerve injury using the SNI model (Cordeiro Matos et al, 2015, Lançon et al, 2021). Analyzing calcium events longitudinally in mice allows us to measure changes in calcium events throughout the onset (P3) and maintenance of chronic pain (P10 – P28), giving us an unparalleled opportunity to determine at which point SNI

induces pathological hyperexcitability. Our results demonstrate that SNI significantly increases the frequency of pain-evoked calcium events but has little effect on their amplitude (Figures 4F and 5D). Following SNI, the frequency of spontaneous calcium events evoked by stimulation of $Na_v1.8$+ nociceptors increased from 0.9 ± 0.016 Hz to 0.17 ± 0.02 Hz, 0.19 ± 0.02 Hz, and 0.24 ± 0.01 Hz at 3, 10, and 28 days post SNI (SNI P3, P10, P28), respectively (two-way ANOVA, p = 0.0072, 0.0034)(Figure 4F). The amplitude of calcium events remained at approximately 2.5 ± 0.1 dF/F z-score from P3 to P28 (Figure 4E).

Similar to $Na_v1.8$ stimulation, the frequency of calcium events evoked by acetone increased from 0.16 ± 0.02 Hz to 0.22 ± 0.03 Hz, 0.27 ± 0.02 Hz, and 0.31 ± 0.03 Hz at SNI P3, P10, and P28, respectively (two-way ANOVA, p = 0.0025)(Figure 5D). As with $Na_v1.8$ stimulation, the amplitude of calcium events was not affected by SNI (Figure 5C).

Given the well documented increase in ACC pyramidal excitability following chronic pain, we also analyzed changes in baseline spontaneous calcium events at SNI P28. Interestingly, we measure a significant increase in the frequency of baseline spontaneous calcium events at SNI P28, but not at P3 or P10 (Figure 4F and 5D). The frequency of calcium events before pain stimulation increased from 0.03 ± 0.01 Hz pre-SNI to 0.11 ± 0.02 Hz at SNI P28 (n = 4 mice, p = 0.0028)(Figure 4F and 5D). Surprisingly, this 2 fold increase in frequency of pain-independent calcium events at SNI P28 now coincides with the frequency of acute pain-induced calcium events pre-SNI (Figure 4F).

SNI Potentiates Pain-Induced Inhibition of DA Release in the ACC

As we measured a substantial increase in calcium event frequency following the onset of SNI, we also investigated if DA release in the ACC is affected. To determine if the onset of chronic pain

affects cortical DA release, we measured noxious stimuli-induced changes in dLight1.1 fluorescence longitudinally in mice before and after SNI. Intriguingly, while acute pain did not decrease ACC DA in naïve mice, we observed significant decreases in DA release in the ACC following acute pain stimulation in mice at P3, P10, and P28 SNI. dLight1.1 fluorescence decreased to -1.64 ± 0.16, -1.61 ± 0.21, and -1.71 ± 0.18 dF/F at SNI P3, P10, and P28, respectively, following ChR2 stimulation of $Na_v1.8$+ nociceptive fibers (two-way anova: p = 0.002 – 0.005)(Figure 4G and 4H).

Similarly to acute pain, acetone-evoked cold pain induced decreases in cortical DA release was also significantly potentiated following SNI. dLight1.1 fluorescence decreased to -2.21 ± 0.29, -2.25 ± 0.33, and -1.86 ± 0.30 dF/F following acetone application, at P3, P10, and P28 SNI, respectively (repeated measures ANOVA: p = 0.009 – 0.02)(Figure 5F). Unlike acute pain which shows a potentiation of peak ACC DA release inhibition all the way through SNI P28, cold pain-evoked peak inhibition of ACC DA release was only significantly potentiated in the early stages of SNI, at P3 – P10, but not at P28 (Figure 4F).

SNI Induces a Prolonged Hypodopaminergic State in ACC Following Pain Stimulation

In addition to the more pronounced decrease in DA release in the ACC following pain stimulation in SNI mice, the time required for DA to return to baseline is significantly increased. Following stimulation of $Na_v1.8$+ nociceptive fibers, the time required for DA levels to return to baseline increases from 4.71 ± 0.91 s in naïve mice to 37.18 ± 2.10 s in mice at SNI P28 (t-test: $t(42) = 11.29$, $P < 0.0001$)(Figure 4I). This SNI-induced prolonged hypodopaminergic state is also observed following noxious cold stimulation. Whereas it required 30.17 ± 2.52 s for DA to return to baseline in naïve mice following acetone application, it required 38.09 ± 1.52 s in mice at SNI P28 (t-test: $t(40) = 2.02$, $p = 0.0251$)(Figure 5I).

Interestingly, this prolonged hypodopaminergic state coincides with pain-induced calcium events. Since dual-color photometry allows us to analyze changes in calcium and DA transients simultaneously, we noticed that the pain-induced prolonged hypodopaminergic state correlates with an increase in calcium events following SNI. This is clearly illustrated when comparing calcium events alongside decreases in DA levels evoked by noxious cold stimulation (Figure 5B, 5H). The correlation between duration of pain-induced hypodopaminergic and frequency of calcium events was found significant ($F(1,52) = 67.2$, $p < 0.0001$)(Figure 5H).

Discussion

The cause of neuropathy-induced hyperexcitability of the ACC is still not well understood despite multiple studies establishing this region as a viable target for the development of novel non-opioidergic pain treatments (Kasanetz et al, 2022; Kuner et al, 2017). Our previous work, and others, establishes DA as a key neuromodulator involved in pathological hyperexcitability in chronic pain and we show here that not only is the D1R-sensitive circuitry in the ACC affected by chronic pain, so is DA release (Lançon et al, 2021).

GABA- and HCN-Mediated Inhibition of Cortical Neurons

Our findings indicate that D1R activation promotes the release of GABA in the ACC, leading to inhibition of pyramidal cells in layer 2/3. This is an additional inhibitory mechanism based on DAergic signaling, independent of the postsynaptic mechanism relying on the D1R-mediated modulation of HCN channe activity that we reported previously (Lançon et al, 2021). We observed that D1R activation exclusively modulates sIPSCs, but not mIPSCs, suggesting that action potentials are necessary for the D1R-mediated facilitation release of GABA. This is in line with

previous reports indicating that D1R agonist are capable of generating action potentials in synaptically isolated fast-spiking (FS+) inhibitory interneurons via inhibition of inward rectifier K^+ and resting leak K^+ currents (Gorelova et al, 2002). The presence of these two independent mechanisms, both relying on D1Rs and inhibiting the same population of neurons, leads us to question their unique cellular and physiological purposes.

Given that HCN channels are primarily expressed on dendrites of cortical neurons and are involved in summation of excitatory post-synaptic potentials (EPSPs), we postulate the role of HCN-mediated inhibition is to regulate the noise threshold of incoming excitatory post-synaptic currents (EPSCs)(Magee 2020). Due to the HCN dysfunction observed in chronic pain, incoming pain-evoked EPSCs are potentiated, converting a subthreshold pain-evoked EPSP, previously filtered out as noise, into an action potential (Cordeiro Matos et al, 2015; Lançon et al, 2021; Santello et al, 2017).

On the other hand, we hypothesize GABA inhibition is used as a tonic inhibitor as well as a compensation mechanism in the event I_h-mediated inhibition is disrupted. Reports indicate that $GABA_A$-mediated inhibition is potentiated in cortical neurons of HCN1-knock out mice (Chen et al, 2010). The loss of I_h is compensated by an increase in $GABA_A$ receptor expression, this tonically increases $GABA_A$ receptor current and results in no net change in dendritic EPSP summation (Chen et al, 2010). Additionally, $GABA_B$-signaling has been shown to modulate the surface expression of HCN1 and HCN2 (Li et al, 2014). In this regard, GABA-mediated inhibition would be a compensatory mechanism and a means to regulate synaptic integration in cortical neurons.

Interestingly, we show D1R-mediated GABA inhibition of layer 2/3 ACC pyramidal neurons is disrupted in neuropathic conditions. Since we observe D1R agonists no longer increase sIPSC

frequency in neuropathic conditions, yet the amplitude of sIPSCs remains unchanged, this attenuation is likely mediated by dysfunction in presynaptic interneurons rather than postsynaptic pyramidal cells (Salin and Prince, 1996). This is in agreement with previous reports demonstrating a synaptic uncoupling between inhibitory interneurons and pyramidal cells in the ACC of neuropathic mice (Blom et al, 2014). Chronic pain induces structural modification in ACC microcircuitry which results in disinhibition (Turget and Altun, 2009; Wang et al, 2005). What causes this reduction of inhibitory synapses on pyramidal cells in chronic pain is not established and merits investigation.

Homeostatic Mechanisms Regulating DA Release in the ACC

Given the robust effect of DA on ACC circuitry, it is critical to understand which homeostatic mechanisms regulate ACC DA release. Since rewarding stimuli are known to activate DAergic cells in the ventral tegmental area (VTA), we tested whether a conditioned sucrose reward affects the release of DA in the ACC (Hernandz and Hoebel, 1988). For the first time, we show convincing evidence that rewarding stimuli promote the release of DA in the ACC, possibly explaining why rewarding stimuli are analgesic by nature (Atlas and Wager, 2012). Additionally, we show that noxious somatosensory stimulation inhibits the release of DA in the ACC. A recent study identified a peripheral pathway responsible for inhibiting VTA DA cell activity following pain stimulation (Yang et al, 2021). This feedforward inhibition stems from the glutamatergic activation of neurons in the lateral parabrachial nucleus (LPB) projecting to the substantia nigra pars reticulata (SNR) or directly to the VTA (Yang et al, 2021). Another study using micro-dialysis to detect DA levels has shown that capsaicin-evoked pain tonically decreases DA release in the nucleus accumbens (NAc)(Gee et al, 2020). In line with these results, we demonstrate here that noxious stimuli inhibit cortical DA release in three different pain models: acute pain via phasic stimulation of peripheral

nociceptive fibers, cold pain evoked by acetone, and chemical pain using intradermal injection of the TRPV1 agonist capsaicin. Furthermore, pain state-evoked decreases in DA release in the ACC are proportional to the intensity and duration of the noxious stimulus. Capsaicin-induced pain, lasting from 3-8 minutes, reduces ACC DA release for 5-8 minutes whereas acetone-induced pain, lasting only 10-15 seconds, reduces ACC DA release for 30 seconds on average.

This robust bidirectional control of ACC DA release by reward and painful stimuli further validates the hypothesis of a hypodopaminergic ACC in chronic neuropathic pain. About ~ 21% of chronic pain patients report depression and major depressive disorder (MDD) is commonly associated with anhedonia, defined as a lack in ability to feel pleasure, as well as a reduction in firing frequency of VTA DAergic neurons (Bair et al, 2003). Chronic pain is also highly prevalent in Parkinsonian patients and, interestingly, L-DOPA supplementation has been shown to decrease pain symptoms and ACC activity (Blanchet et al, 2018; Brefel et al, 2005; Defazio et al, 2008).

Cortical Excitability and DA Release in Chronic Pain

To test if DA release in the ACC is impacted by chronic pain and whether these alterations influence pyramidal cell excitability, we recorded pyramidal calcium events in conjunction with DA release following the onset of neuropathic nerve injury. We show pain stimulation reliably produces time-locked calcium events in the ACC, likely mediated by glutamatergic inputs from the medial thalamus (Meda et al, 2019; Xue et al, 2022). During the acute inflammatory stages of neuropathic injury (SNI P3), acetone and acute pain-evoked pyramidal calcium events were potentiated and the inhibition of DA release was increased. Interestingly, although acute stimulation of nociceptive fibers did not produce a significant reduction in DA release pre-SNI, we measured a substantial decrease in DA following nerve injury. This potentiation could be the result of peripheral sensitization during the early stages of nerve injury, where this acute form of

pain stimulation was below the required threshold to inhibit VTA neurons pre-SNI. Conversely, it is possible dLight1.1 is not sensitive enough to detect sharp, phasic, decreases in DA release induced by this type of pain stimulation.

As the acute stages of inflammatory pain ended and the maintenance stage began (P10-P28), we noticed several changes in calcium events and DA release in the ACC. First, both pain-evoked and baseline calcium events were significantly increased. This coincides with the onset of the typical pathological cortical hyperexcitability observed in chronic pain. Secondly, whereas pain produced an acute drop in DA release pre-SNI, we noticed a prolonged hypodopaminergic state following pain stimulation in neuropathic mice. The time required for extracellular DA to return to baseline values pre-pain stimulation was drastically increased during the maintenance period of chronic pain. Coincidentally, this prolonged hypodopaminergic state is correlated to the frequency of calcium events following pain stimulation. These results indicate chronic pain significantly potentiates both pyramidal calcium events and pain-induced inhibition of DA release in the ACC.

As chronic pain is associated with GABA-mediated decreases in DAergic neuron activity in the VTA, this prolonged hypodopaminergic state could be caused by persistent inhibition in neuropathic VTA (Huang et al, 2019; Ko et al, 2018). The mu-opioid receptor (MOR) is expressed on GABAergic interneurons in the VTA and their activation is well known to promote the release of DA via inhibition of GABAergic interneurons (Narita et al, 2001). Since MOR activation in VTA is decreased in chronic pain, this interaction could be responsible for mediating the increased inhibition of cortically projecting DAergic cells in the VTA (Ozaki et al, 2002) Similarly, the G_i-coupled kappa opioid receptor (KOR) is expressed on DA terminals and involved in modulating DA release. As an upregulation of prodynorphin mRNA, the precursor to the endogenous KOR

agonist dynorphin, has been reported in the neuropathic ACC, pathological KOR signaling could be involved in decreasing cortical DA release (Palmisano et al, 2019).

Although further research is required to determine how chronic pain affects circuits that promote DA release, such as the reward pathways, it has been reported that pain relief no longer elicits DA release in the late stages of neuropathic pain (Kato et al, 2016).

Conclusion

We demonstrate here that pain and reward bidirectionally control the release of cortical DA, and the onset of chronic pain significantly affects DA release in the ACC. In addition to our previous work that establishes supplementing supraspinal DA reduces chronic pain symptoms and cortical hyperexcitability, these results further support the idea that targeting the mesocortical DA pathway can be a viable strategy to treating chronic pain.

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Contributions: Experiments were performed by KL. KL and PS designed the project and wrote the manuscript.

Declaration of Interests: None to report.

Figure and Legends

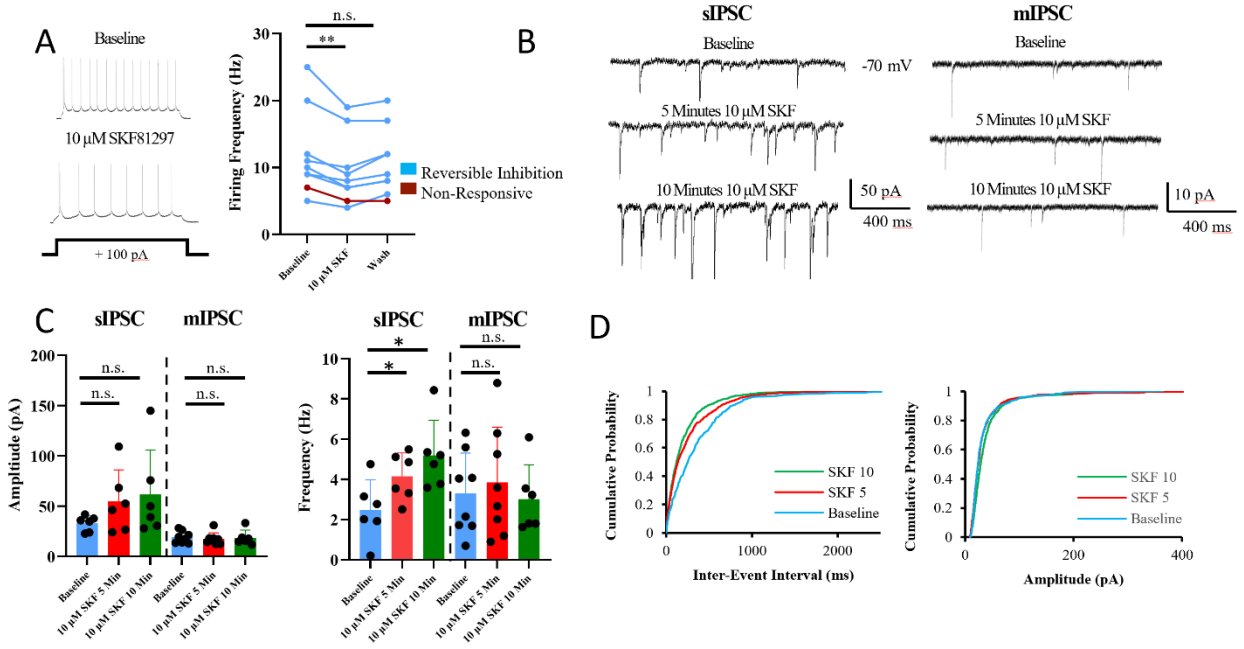

Figure 1. D1R activation in the ACC inhibits pyramidal neurons by promoting GABA release.

(A) Following application of 10 µM SKF81297, a large majority of layer 2/3 ACC pyramidal neurons display reversible inhibition (n = 9).

(B) Voltage clamp electrophysiology recordings of IPSCs in the presence (right, mIPSC) or absence of TTX (left, sIPSC) following application of 10 µM SKF81297 for 5 and 10 minutes. 10 µM SKF81297 increases sIPSCs but not mIPSCs.

(C) Group analysis of change in amplitude (left) and frequency (right) of sIPSC and mIPSCs induced by 10 µM SKF81297. SKF81297 significantly increases frequency but not amplitude of sIPSC, with no effect on either frequency or amplitude of mISPCs (n = 6-9).

(D) Cumulative probability plots show 10 µM SKF81297 significantly decreases inter-event intervals (left) but not amplitude of events (right) in sIPSCs (n = 6-9).

Values represented as means ± SEM or individual values ± SEM: *$p < 0.05$, **$p < 0.01$.

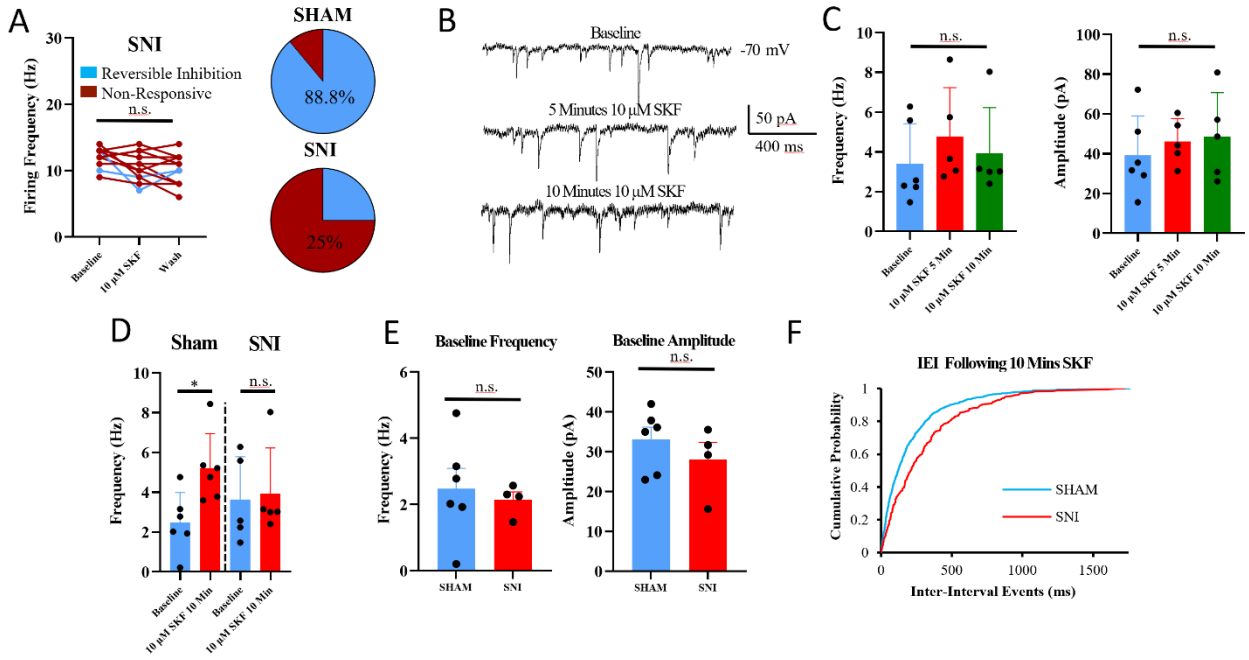

Figure 2. SNI induces a decrease in D1R-induced GABA inhibition of pyramidal firing frequency

(A) 10 μM SKF81297-induced GABAergic inhibition of ACC layer 2/3 pyramidal neurons is diminished in neuropathic mice. Right: voltage-clamp electrophysiology traces and left: group analysis (n = 12)(left). Proportions of pyramidal neurons responding to SKF81297 in sham and SNI mice (right).

(B) Voltage clamp electrophysiology recordings of sIPSCs in SNI mice. SKF81297-evoked increase in sIPSCs frequency is absent in SNI neuropathic mice (n=5-6 per group).

(C) Quantitative comparison of 10 μM SKF81297's effect on sIPSC frequency in SNI vs sham mice (n = 5-6 per group).

(D) Quantitative comparison of SKF81297's effect on sIPSC frequency in SNI and sham mice. No significant difference in sIPSC frequency (left) or amplitude (right) following 10 minute application of 10 μM SKF81297 (n = 5-6 per group).

(E) Quantitative comparison of baseline sIPSC frequency and amplitude between SNI and sham mice (n = 5-6 per group).

(F) Cumulative probability plots illustrating difference in SKF81297 evokved change in sIPSC inter-event interval and amplitude between SNI and sham mice.

Values represented as means ± SEM or individual values ± SEM: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Values represented as means ± SEM and individual values ± SEM: *p < 0.05.

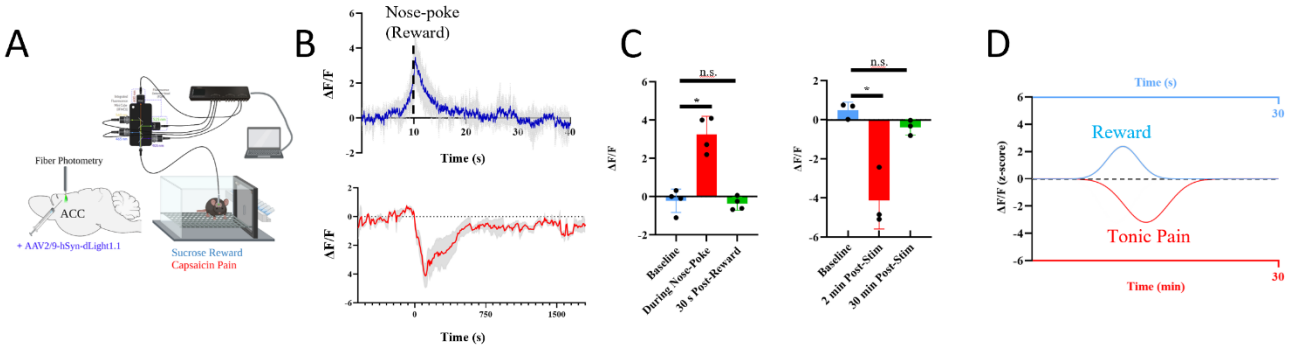

Figure 3. Pain and reward bidirectionally control release of DA in ACC

(A) *In vivo* real-time detection of reward and pain-evoked changes in DA release in the ACC of freely moving naïve mice in operant 15% sucrose conditioning and 1% capsaicin paradigm.

(B) Reward seeking behavior (nose-poke) causes a release of DA in ACC of naïve mice (top) (n= 4 mice, 40-95 trials per mouse). Tonic pain induced by 1% capsaicin injected i.d. in the plantar section of the foot induces a decrease in DA release in the ACC of naïve mice (bottom) (n= 3 mice, 1-3 trials per mouse).

(C) Quantitative group analysis demonstrates significant changes in ACC DA dLight1.1 fluorescence following reward (left) and pain (right) (n = 1-4 mice per group).

(D) Nonlinear regression curve fit illustrates reward and pain modulates bidirectionally DA release in the ACC.

Values represented as means with individual animals ± SEM : *p < 0.05.

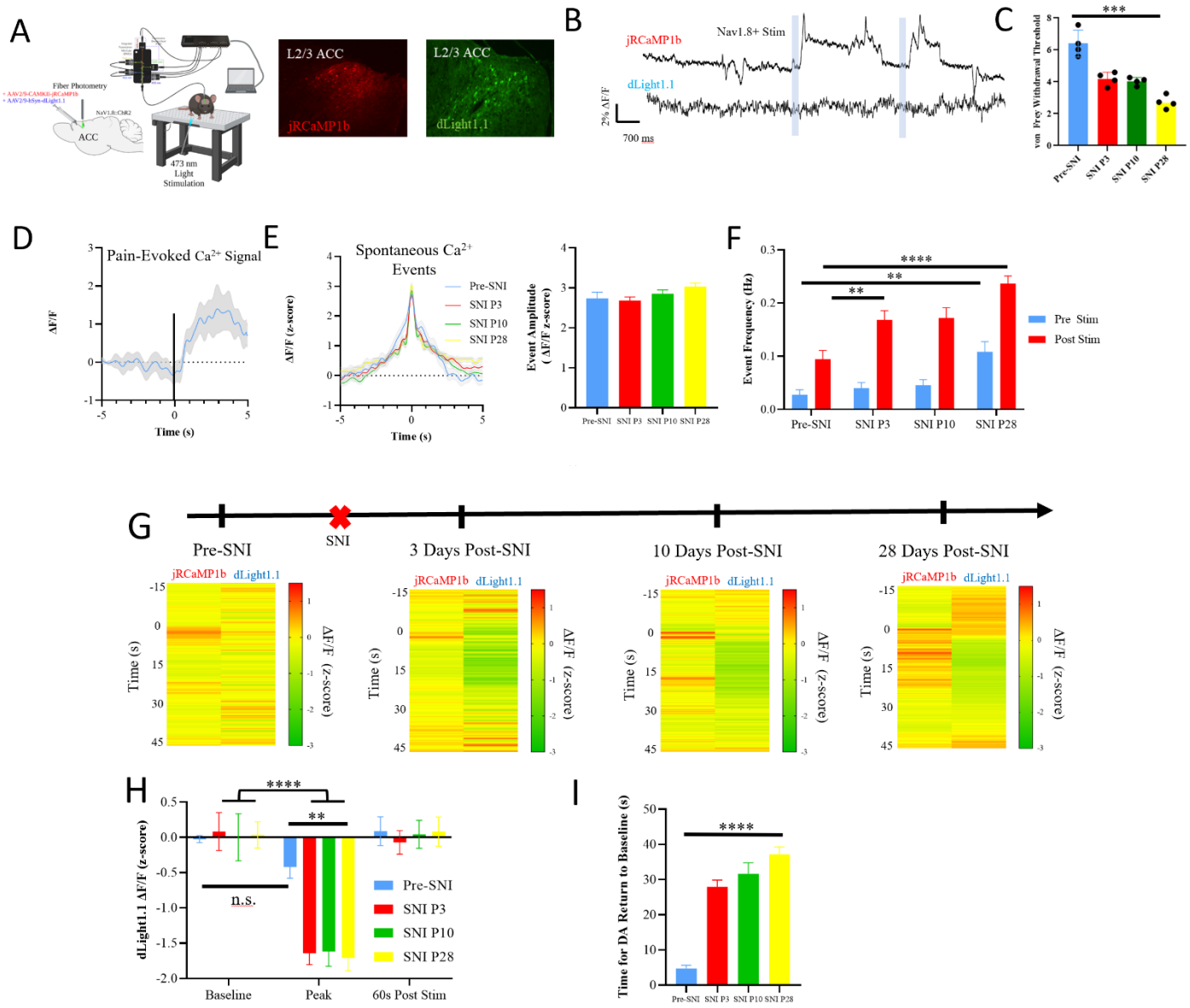

Figure 4. SNI increases acute pain-induced inhibition of DA release and calcium event frequency in the ACC

(A) Paradigm for *in vivo* real-time detection of calcium and dopamine transients, using AAV2/9-CAMKII-jRCaMP1b and AAV2/9-hSyn-dLight1.1 injected in the ACC, in freely moving mice following acute pain evoked by stimulation of Nav1.8+ peripheral fibers using ChR2 (left). Confocal images of ACC layer 2/3 showing expression of jRCaMP1b and dLight1.1 (right).

(B) Individual sample traces showing changes in fluorescent of, both jRCaMP1b and dLight1.1, following acute pain induced by activation of peripheral Nav1.8+ fibers.

(C) Ipsilateral mechanical withdrawal values of mice following SNI (n= 4 mice).

(D) Average time-locked response of jRCaMP1b fluorescence in the ACC following peripheral stimulation of Nav1.8+ fibers using ChR2 (n = 6 mice, 5-10 trials per mouse).

(E) Amplitude of detected calcium events in ACC evoked by optopain stimulation in mice pre-SNI, 3 days post-SNI, 10 days post-SNI, and 28 days post-SNI (left). Quantitative group analysis reveals no significant difference in event amplitude following SNI (right) (n = 4-6 mice, 32-164 events per group).

(F) Quantitative analysis comparing the frequency of calcium events before and after optopain stimulation in mice pre-SNI, 3 days post-SNI, 10 days post-SNI, and 28 days post-SNI. SNI significantly increased the frequency of calcium event at 3 and 10 days (n = 4-6 mice, 16-37 trials per group).

(G) Heat maps illustrating a significant increase in pyramidal calcium events (jRCaMP1b) and decrease in DA release (dLight1.1) following acute pain stimuli in mice pre-SNI, 3 day post-SNI, 10 days post-SNI, and 28 days post-SNI (n = 4-6 mice, 16-37 trials per group).

(H) SNI onset significantly increases acute pain-induced reduction in DA release in the ACC at 3 day post-SNI, 10 days post-SNI, and 28 days post-SNI (n= 4-6 mice, 16-37 trials).

(I) SNI increases the duration of hypodopaminergic state in the ACC when evoked by acute pain (n= 4-6 mice, 16-37 trials).

Values represented as dF/F or dF/F (z-score) with means ± SEM: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

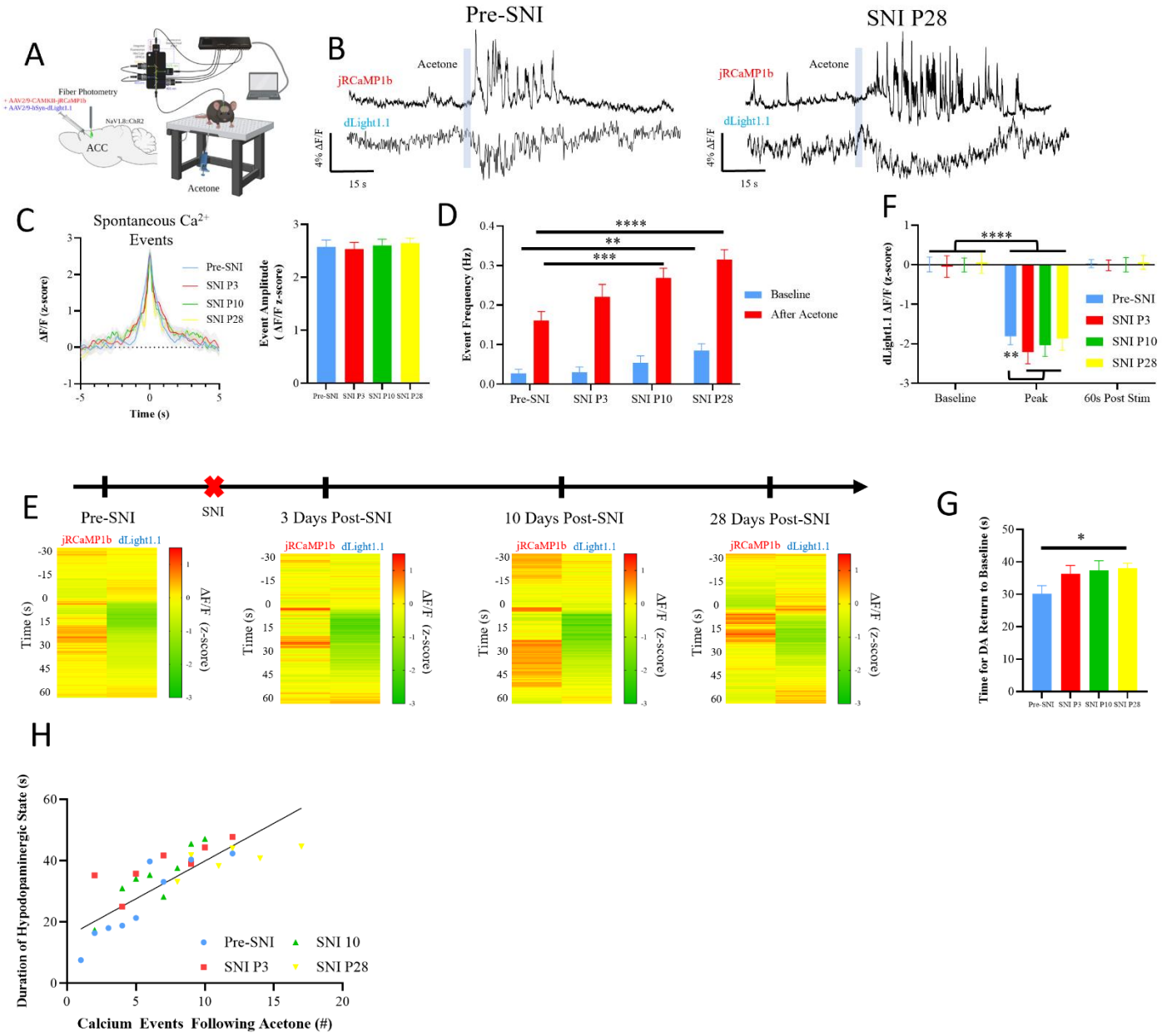

Figure 5. SNI increases cold pain-induced inhibition of DA release and calcium event frequency in the ACC

(A)Paradigm for *in vivo* real-time detection of calcium and dopamine transients, using AAV2/9-CAMKII-jRCaMP1b and AAV2/9-hSyn-dLight1.1 injected in the ACC, in freely moving mice following acetone-induced cold pain.

(B) Individual sample traces showing changes in fluorescence signals of both jRCaMP1b and dLight1.1 following application of acetone to induce cold pain in mice pre-SNI (left) and at SNI P3 (right).

(C) Amplitude of calcium events evoked by acetone pain stimulation pre-SNI, 3 days post-SNI, and 10 days post-SNI (left). Quantitative group analysis reveals no significant difference in event amplitude following SNI (right) (n = 4-6 mice, 65-129 events per group).

(D) Quantitative analysis comparing the frequency of calcium events before and after cold pain stimulation in mice Pre-SNI, SNI P3, SNI P10, and SNI P28 (n = 4-6 mice, 19-40 trials).

(E) Heat maps illustrating the significant increase in pyramidal calcium (jRCaMP1b) and decrease in DA (dLight1.1) following cold pain stimuli in mice pre-SNI, 3 days post SNI, 10 days post SNI, and 28 days post SNI (n = 4-6 mice, 19-40 trials).

(F) SNI onset significantly alters cold-pain induced decreases in ACC DA release (n =4-6 mice, 19-40 trials).

(G) SNI increases the duration of a hypodopaminergic ACC evoked by acetone cold pain (n =4-6 mice, 19-40 trials).

(H) Correlation between hypodopaminergic ACC evoked by acetone and the number of calcium events following acetone stimulation (n = 4 mice).

Values represented as dF/F or dF/F (z-score) with means ± SEM: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
AAV2/9-CAMKII-jRCaMP1b	Canadian Neurophotonics Platform	AAV1935
AAV2/9-hSyn-dLight1.1	Canadian Neurophotonics Platform	AAV1721
Chemicals, peptides, and recombinant proteins		
SKF81297	ToCris	Cat#1447
TTX	Abcam	ab120055
Kynurenic Acid	Millipore-Sigma	Cat# K3375
Experimental models: Organisms/strains		
Wild-Type C57Bl6 Mice	Charles River Canada	C57Bl6
ChR2-EYFP Mice	Jackson Laboratory	Ai32
Nav 1.8-Cre	Jackson Laboratory	JAX#036564
Software and algorithms		
pClamp	Molecular Devices	RRID:SCR_011323
Doric Neuroscience Studios	Doric Lenses	N/A
Prism 9	Graphpad	RRID:SCR_002798
NIS-Elements Viewer	Nikon	N/A
Minianalysis	Synaptosoft	N/A
Matlab v9.9	Mathworks	N/A
Other		
473 nm DPSS Laser	LaserGlow	R471005GX
300 μm core optical fiber	ThorLabs	FT300UMT
Custom Photometry Patch Cord	Doric Lenses	MFP_400/430/1100-0.57_0.85m_FCM-MF1.25_LAF
Vetbond	3M	1469SB
0.6 mm Drill Bit	SGS	07283
Stainless Steel Screw	J.I. Morris	NAS723CE100-100
200 μm core optical fiber	ThorLabs	FG200UCC

STAR Methods

Experimental Model and Subject Details

All animals were housed in the Montreal Neurological Institute (MNI) Animal Care Facility (ACF) and all procedures are following the Canadian Council on Animal Care guidelines. 6 week old male C57BL/6 mice (Charles River Laboratories, QC, CA) were used for electrophysiology experiments. Transgenic male $Na_v1.8^{ChR2\text{-}EGFP}$ mice were bred in house and were used for behavioral experiments at 8-9 weeks of age. All mice were kept in a 12 hour light-dark cycle with free access to water and food.

Methodological Details

Spared Nerve Injury (SNI)

Spared nerve injury (SNI) surgery to induce chronic neuropathic pain was performed on C57BL/6 mice at 6 weeks of age, 21 days before electrophysiology recordings, and on $Na_v1.8^{ChR2\text{-}EGFP}$ mice at 9 weeks of age, 3 weeks following optical fiber implantation. 2% isoflurane was used to anesthetize mice. The tibial and peroneal nerves were ligated and cut, leaving the sural nerve intact.

Electrophysiological Recordings

Animals were anesthetized with an Avertin solution (2.5 g tribromoethanol in 5 mL amylene hydrate diluted in 100 mL $_{dd}H_2O$) (Sigma-Aldrich, St. Louis, MI). Then animals were transcardically perfused with a 4^oC choline-chloride based cutting solution oxygenated with carbogen (O_2 95%, CO_2 5%) (Praxair). Brains were extracted and sliced into 300 µm sections using a vibratome (Leica VT1000). Slices were allowed to rest at room temperature for 1 hour in oxygenated (see above) artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 2 mM KCl, 26 mM $NaHCO_3$, 1.8 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 10 mM Glucose, 1.6 mM $CaCl_2$, pH 7.4. Slices were submerged at 30-32^oC on the stage of a Zeiss Axioskop microscope continuously

perfused with oxygenated (see above) aCSF containing 1.8 mM Kynurenic Acid at a rate of 1 mL/minute. A near-infrared CCD camera coupled to a x63 water immersion objective was used during patch-clamp recordings (Sony XC-75). Cells were patched with BF150-75-10 glass pipettes (~ 6 MΩ) pulled with a Flaming Brown Micropipette Puller (Model P-97, Sutter Instruments, US). Pipettes were mounted on a MP-225 micromanipulator and filled with an intracellular solution containing 120mM K-gluconate, 10 mM HEPES, 0.2 mM EGTA, 20 mM KCl, 2 mM $MgCl_2$, 7 mM diTrisPhosphate-Creatine, 4 mM Na_2ATP, and 0.3 mM NaGTP (Sutter Instruments, Novato, CA). An Axopatch 200B amplifier and Digidata 1322A interface digitizer were used for data acquisition (Molecular Devices, San Jose, CA). Signals were low-pass filtered at 10 kHz for current-clamp recordings and at 2 kHz for voltage-clamp recordings, digitized at 20 kHz.

Neurons were patched in areas corresponding to layer 2/3 in dACC according to stereotaxic coordinates (Paxinos et al, 2008). Pyramidal cells were identified based on firing frequency, input resistance (MΩ), and spike adaptation to a 1-second 100 pA cuurent injection pulse. Cells with resting membrane potentials (RMP) above -50 mV or below -80 mV were excluded. Series resistance was compensated (≤ 35 MΩ). Firing rate (Hz) was evaluated in current clamp mode by bringing cells to -60 mV RMP and counting action potentials evoked by a +100 pA current pulse.

mIPSCs and sIPSCs were measured in voltage clamp mode using a CsCl internal solution to block post-synaptic potassium channels. 1.8 mM kynurenic acid was added to the extracelullar solution to block fast synaptic transmission. mIPSCs were recorded in the presence of 1 µM TTX to block voltage-gated sodium channels. Cells were held at a holding potential of -70 mV, sIPSCs and mIPSCs were analyzed using MiniAnalysis (Synaptosoft).

Changes in sIPSC and mIPSC amplitude and frequency were assessed by obtaining baseline values 5 minutes after patching and comparing them with values obtained following 5 and 10-minute treatment with 10 µM SKF81297. Resting membrane potential (mV), temperature, series resistance (MΩ), and whole-cell capacitance were measured before each time point.

Intracranial Optic Fiber Implantation and Virus Injection

Stereotaxic optic fiber implantation surgeries were performed on 5-6 week old male $Na_v1.8^{ChR2\text{-}EGFP}$ and C57BL6 mice anesthetized with a 2% isoflurane. Topical 2% xylocaine (AstraZeneca) and 5 mg/mL carprofen (Zoetis, 20 mg/kg) s.c. were administered prior to surgery. The skull was fixed and leveled using a stereotaxic frame (Kopf Instruments). A 0.6 mm drill bit (07283, SGS) was used to drill three holes in the skull, two were used for implantation of small stainless steel screws (NAS723CE100-100, J.I. Morris) to stabilize the head cap and another pertaining to the injection and implantation of the virus and optical fiber in the ACC, respectively.

For dual-color photometry experiments, 500 nL of AAV2/9-CAMKII-jRCaMP1b and AAV2/9-hSyn-dLight1.1 viral particles (titer= 1-2E13 gc/ml) were co-injected unilaterally at a rale of 100 nL/min in the ACC (distance from Bregman: +1.3 mm AP, ±0.3 mm ML, - 1.8 mm DV). For single color dLight1.1 experiments, 1 µL AAV2/9-hSyn-dLight1.1 was injected instead. Viruses were injected using a 1 µL Neuros Hamilton syringe. Viruses used were titrated at 2.0×10^{13} GC/mL and acquired from the Canadian Neurophotonics platform (CNP).

After virus injection, a 300 µm diameter optical fiber (0.39 NA FT300UMT, ThorLabs) was implanted in the ACC, 0.1 mm above the injection site. The head screws and optical fiber implant were fixed to the skull of the mice using C&B Metabond and dental cement (Patterson Dental). To prevent infection, Vetbond (3M) was then used to attach the surrounding skin to the head. capMice

then received daily carprofen (s.c., 20 mg/kg) for 3 days following surgery and were allowed to recover for 14 days before photometry behavioral conditioning started.

Photometry Recordings

Photometry recordings were performed using a 1-site, 2-color system from Doric Lenses. Isobestic fluorescence (calcium and dopamine insensitive) was measured with a 405 nm LED oscillating at 208 Hz. Calcium-sensitive fluorescence (jRCaMP1b) and dopamine-sensitive fluorescence (dLight1.1) were measured with 470 nm and 568 nm LEDs oscillating at 470 Hz and 333 Hz, respectively. LED intensities fluctuated between 20 and 200 µW. The recorded signal was digitized at 6 kHz and the demodulated signals were low-pass filtered at 12 Hz. A custom build 0.85 m 400 µm patch cord was used to deliver and collect light from the implant (0.59 NA, MFP_400/300, Doric Lenses). Least squared linear regression was used to best fit the 405 nm control signal to the 470 nm and 568 nm signals and to calculate dF/F.

Photometry signals were time-locked to stimuli and average across mice for each given stimuli. Data decimated by a factor of 200 and processed using a custom Matlab (MathWorks) script. Photometry signals were analyzed in 90 s windows, with the pain or reward stimuli time-locked at 30 s. Frequency and amplitude of calcium events were analyzed for baseline (30 s window before stimulus) and following pain stimulation (60 s window post stimulus). Due to the nature of these recordings, data with large movement artifacts were omitted. Acute pain stimulations were kept at a minimum of 5 minutes apart to avoid potential overlap with previous pain stimulations.

Behavioral Tests

For all behavioral recordings, mice were first habituated to the recording chamber, experimenter, and the patch cord sensation for 1 week. Behavioral tests were always performed during their usual light cycle, from 10 am to 4 pm, with the same experimenter.

Von Frey: Animals were placed in suspended chambers with wire mesh floors for 30 minutes for short-term habituation prior to testing. Mouse withdrawal thresholds were determined by stimulating the lateral planar surface of both the ipsi- and contralateral paws using an automatic von Frey apparatus (Ugo Basile, Italy).

Acetone and capsaicin stimulation: Cold pain was triggered by applying 30 µL of acetone to the lateral planar surface of the paw. Tonic pain was simulated by using 10 µL 1% capsaicin (Millipore Sigma). Capsaicin was dissolved in ethanol and intradermally (i.d.) injected under the lateral planar surface of the ipsilateral paw using a 10 µL Hamilton syringe. To avoid fear conditioning and habituation, acetone was applied in 10 minute intervals, with a maximum of 5 applications per recording session and capsaicin was only applied one time per session. Only one type of pain stimulation was used per recording session.

$Na_v1.8$ stimulation: A 473 nm diode-pumped solid-state (DPSS) laser (max 120 mW, LaserGlow) was used for selective ChR2 activation in $Na_v1.8^{ChR2\text{-}EGFP}$ mice to induce acute pain. Light was shone on the lateral planar surface of the ipsilateral paw at 7.5-8.5 mW using a 200 µm patch cord (FG200UCC, Thorlabs), at a distance of 5-10 mm from the skin. A TTL pulse train generator (Prizmatix) was used to generate 300 ms laser pulses and to time-lock the pain stimulus to the photometry recordings. $Na_v1.8$ pain stimulation was evoked in 5 minute intervals, with a maximum of 5 applications per recording session.

Sucrose conditioning and reward: 2 weeks following optic fiber implant surgery, 8-9 week old male C57BL6 mice were placed on a restricted feeding schedule. The weight of these mice was monitored daily and food availability was restricted to maintain them at 85%-90% of pre-surgery body weight. A daily feeding and weight log was kept for the duration of the experiment. Mice were then conditioned in an operant conditioning chamber (Med Associates) with access to a lever

for 40 minute sessions. The lever was presented for 10 s in 30 s intervals. Successful lever presses resulted in a auditory tone (4.8kHz, 80dB, 5s duration) and dispensing 30 µL of a 15% sucrose solution (m/v). The photometry signal was time-locked to lever presses and nose pokes, indicating when they received the sucrose reward, using a RZ5P signal processor (Med Associates).

Drugs and Solutions

Reagents used for aCSF, cutting solution, internal pipette solution, and capsaicin were purchased from Sigma-Aldrich (St. Louis, Missouri). SKF81297 was obtained from Tocris (Bristol, UK). TTX was obtained from Abcam (Cambridge, UK). Carprofen was obtained from All drugs were diluted and aliquoted into single-use samples stored at -20^{o}C.

Quantification and Statistical Analysis

Electrophysiological data were analyzed using Clampfit 10 and statistical analysis was performed using GraphPad Prism 9. Values are represented as individual values or means ± SEM with significance threshold set at $p < 0.05$. One-way ANOVA with Dunnett's multiple comparison was used to compare multiple independent groups (e.g. SNI vs SHAM mIPSC frequency). Unpaired t-tests were used to compare two independent groups whereas the paired t-test was used to compare two related groups (baseline vs treatment). Two-way ANOVA with multiple comparison was used to analyze significance between independent samples under multiple conditions.

Photometry data was analyzed using Doric Neuroscience studios, GraphPad Prism 9, and Matlab. A custom script was used for baseline correction, normalization, and motion correction of the calcium and dopamine-sensitive signals relative to the 405 nm control signal. Calcium events were detected at 2 standard deviations away from the mean and averaged for frequency and amplitude analysis (code generated by Leonardo Molina, University of Calgary). For illustrative purposes,

group averaged signals were best fit with a nonlinear regression curve fit. Graphpad Prism 9 was used for further statistical analysis and heat-map generation.

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Chapter 4

Summary

Discussion

4.1 Physiological Roles of HCN- and GABA-mediated inhibition of the ACC

4.2 Potential mechanisms responsible for altering ACC DA release in chronic pain

4.3 The role of opioids in dysregulating mesocortical DAergic signaling

4.4 Pain relief requires ACC D1R signaling

4.5 Therapeutic relevance

Limitations

4.6 Sex-specific differences

4.7 The innate problem with photometry

Future Directions

Conclusions

Summary

This summary provides a brief overview of the results presented in Chapter 2 and 3, as well as how these results reach the objectives of this dissertation.

Objective 1: Determine DA's neuromodulatory effect in the anterior cingulate cortex and its involvement in top-down pain processing.

The *in vitro* electrophysiology results obtained in Chapter 2 establish that DA is a robust neuromodulator of pyramidal excitability in the ACC. This inhibition is mediated by the G_s-coupled D1 receptor, whose activation increases intracellular cAMP and promotes the opening of HCN channels. Pharmacological blockade of HCN channels occludes DA's inhibitory effect on synaptically-isolated pyramidal neurons, indicating DA-mediated inhibition is dependent on HCN channel opening.

Furthermore, we show HCN-mediated inhibition is unaffected by chronic pain. Since the post-synaptic mechanism that mediate DA's inhibitory control of pyramidal neurons is preserved in neuropathic conditions, a decrease in mesocortical DA signaling could underlie the pyramidal hyperexcitability in chronic pain. Accordingly, we show that increasing supraspinal DA via L-DOPA attenuates chronic pain-induced increases in pain sensitivity and cortical hyperexcitability.

Concerning the role of ACC DA in modulating top-down pain processing, we provide evidence that intracortical injection of D1R agonists decreases pain perception and induces positive CPP. Moreover, we show that D1R signaling in the ACC is necessary for effective pain relief. These results conclusively prove that DA modulation of the ACC is critically involved in mediating pain perception.

Objective 2: To investigate which homeostatic mechanisms mediate DA release in the ACC and whether they are affected by the onset of chronic pain.

In Chapter 3 we identified a novel mechanism contributing to DA's modulatory role in the ACC. Activation of D1R increases the frequency of inhibitory post-synaptic currents on pyramidal cells in the ACC. Interestingly, we show evidence that this pathway is severely impacted following neuropathic pain (SNI model). D1R activation in neuropathic mice was unable to increase inhibitory currents, leading to a loss of D1R-mediated inhibition in the ACC.

Using the GENI dLight 1.1 and the GECI jRCaMP1b, in Chapter 3 we also demonstrate that pain and reward bidirectionally control the release of DA in the ACC. Rewarding stimuli, such as sucrose, increases cortical DA release while noxious stimulation decreases it. Furthermore, we show the onset of chronic pain significantly alters pain-induced inhibition of DA release in the ACC. 28 days after nerve injury, noxious stimuli-induced inhibition of DA release was potentiated, leading to a prolonged hypodopaminergic state. Intriguingly, this prolonged hypodopaminergic state is correlated to a potentiation of pain-induced calcium transients in ACC pyramidal neurons. Chronic pain increased the frequency of ACC pyramidal calcium events induced by both acute pain, evoked via optogenetic stimulation of peripheral nociceptive fibers, and cold pain, evoked by application of acetone on the hind paw. These results establish that chronic pain conditions promote a dysregulation of mesocortical DAergic signal and this dysregulation is tied to the potentiation of pyramidal excitability in the ACC.

Discussion

Below is a brief discussion explaining the findings of Chapters 2 and 3, their implications for understanding the dysregulated cortical circuits induced by chronic pain, and the prospects for the development of novel treatment options for chronic pain patients.

4.1 Physiological Roles of HCN- and GABA-mediated inhibition of the ACC

In Chapters 2 and 3 we identified two independent, convergent, D1R-mediated mechanisms responsible for inhibiting pyramidal neurons in the ACC. One mechanism is postsynaptic and involves the D1R-mediated opening of HCN channels in pyramidal cells. We show that postsynaptic D1R activation increases I_h by increasing the open channel probability of HCN channels at physiological potentials. This causes a decrease in input resistance and consequently pyramidal excitability. The other mechanism is GABA-dependent and is tied to the activation of D1R on presynaptic interneurons. D1R agonists facilitates the release of GABA, causing tonic inhibition in pyramidal cells in the ACC.

The existence of these two convergent inhibitory mechanisms begs the question: is one of these pathways redundant or do they each have their own unique physiological roles? Although we cannot conclusively answer this question with the results presented here, we can speculate multiple answers. HCN channels are expressed on the dendrites of pyramidal neurons and are primarily involved in dendritic summation of excitatory post-synaptic potentials (Day et al, 2005; Magee 2000). A decrease in HCN open channel probability will insulate dendrites and decrease the threshold required to elicit an action potential. The ratio of G_s/G_i-signaling plays a large role in dictating the open channel probability of HCN channels. When the ratio of postsynaptic G_s- to

G_i-signaling is elevated, via increases in extracellular DA and 5-HT levels for example, intracellular cAMP signaling is increased and the input resistance of dendrites is reduced, increasing the threshold required to elicit an action potential (Lançon et al, 2021; Santello et al, 2017). Conversely, when the ratio of G_s- to G_i-signaling is decreased, via decreases in extracellular DA and increases in NE, pyramidal cells show increased excitability and a reduction in current required to elicit an action potential (Zang et al, 2013). In this sense, dendritic HCN can be viewed as a mechanism to suppress noise, or sub-threshold events, in circuits of the ACC.

The physiological ramifications of this mechanisms can be seen in patients with chronic pain and other disorders with working memory impairments, one of the ACC's main functions (Berryman et al, 2013; Cai and Arnsten, 1997).The Arnsten group has repeatedly demonstrated that pharmacological intervention of this mechanism, via DA or NE agonists, has an irrefutable impact on working memory and attention performance in primates and several other species (Cai and Arnsten, 1997; Mao et al, 1999). Activation of α2 adrenoceptor agonists potentiates incoming signals while activation of D1R decreases them (Brennan and Arnsten, 20008). Since D1R activation increases the threshold required to elicit an action potential, increases in prefrontal DA signaling results in the filtering out of relevant information and impairs working memory. Conversely, decreases in NE signaling is also associated with working memory impairments via the same mechanism and relevant information is filtered out as noise (Brennan and Arnsten, 2008). This mechanism is hypothesized to underlie the cognitive deficits in attention-deficit hyperactivity disorder (ADHD).

Going back to chronic pain, the pathological changes in cortical NE and DA signaling is likely promoting a similar, yet inversed result. Decreases in cortical DA causes irrelevant information, previously filtered out as noise, to be amplified. The same effect can be observed

with increases in cortical NE signaling and this mechanism is thought to underlie the high prevalence of working memory deficits in chronic pain patients. In terms of pain perception, the HCN dysfunction present in chronic pain is likely converting subthreshold irrelevant events into physiological signals, potentially changing the perception of an innocuous stimulus into a noxious one (allodynia). This could also contribute to the high occurrence of spontaneous pain present in chronic pain patients (Scholz et al, 2019). In short, the role of dendritic HCN channels in the prefrontal cortex seems directly tied to mediating the threshold required for a given stimulus to draw and maintain our attention.

Although the physiological role for GABA-mediated inhibition of ACC pyramidal cells is more elusive, reports indicate it could be a compensatory mechanism for changes in HCN expression. Downregulation of HCN1 increases the expression of $GABA_A$ receptors, resulting in no net change in dendritic EPSP summation (Chen et al, 2010). Furthermore, evidence links $GABA_B$ signaling to the surface expression of HCN channels (Li et al, 2014). Given this information, the physiological impact of the HCN channel dysfunction of present in neuropathic conditions should be attenuated by GABA inhibition. However, we show that GABA inhibition is decreased in chronic pain, which we hypothesize is mediated via the synaptic uncoupling of pyramidal cells and inhibitory interneurons (Blom et al, 2014). Thus, if the HCN dysfunction in chronic pain induces an increase in pyramidal $GABA_A$ expression, it is unlikely to increase $GABA_A$ signaling as GABA is quite simply not present.

In conclusion, we postulate that HCN- and GABA-mediated inhibitions are synergistic, yet physiologically distinct, however both contribute to DA's robust neuromodulatory effect on the cortical circuits of the ACC.

4.2 Potential mechanisms responsible for altering cortical DA release in chronic pain

Acute pain has been shown to decrease phasic DA release in the mesolimbic pathway via inhibition of the firing activity of DAergic projection neurons in the VTA (Isakov et al, 2017). However, the impact of chronic pain on DA release in the ACC has yet to be investigated. Our photometry results in Chapter 3 demonstrate that chronic pain significantly alters DA release in the ACC. The cause of chronic pain-induced alterations in mesocortical DA release is not established, but reports indicate this is likely mediated by pathological alterations in the inhibitory circuitry in the VTA. Multiple studies have shown that chronic pain reduces the excitability of DAergic neurons in the VTA (Huang et al, 2019; Ko et al, 2018). VTA GABAergic inhibitory interneurons play a critical role in modulating DA release along the mesocortical and mesolimbic pathways (Nieh et al, 2016). Accordingly, hyperactivity of these inhibitory interneurons is highly associated with anxiety, pain and mood behaviors (Kim et al, 2016; van Zessen et al, 2012). Following nerve injury, the excitability of VTA GABAergic neurons is increased, resulting in higher concentrations of extracellular GABA and pathological tonic inhibition of DAergic projection neurons (Ko et al, 2018).

The cause for this increase in GABA-mediated inhibition of VTA DAergic cells in chronic pain remains unknown, but ineffective inhibition of inhibitory interneurons in the VTA by opioids is a prime suspect (Taylor et al, 2016).

4.3 The role of opioids in dysregulating mesocortical DAergic signaling

The opioidergic system is integral in mediating pain perception, via its effects in both the central nervous system and the periphery, and is well known for its robust control over supraspinal DAergic pathways. 30-70% of GABAergic inhibitory interneurons in the VTA express the mu

opioid receptor (MOR), a G_i-coupled receptor sensitive to the endogenous release of enkephalin and endorphins (Galaj et al, 2020). Activation of MORs expressed on these interneurons disinhibits DAergic cells in the VTA and facilitates the release of DA (Narita et al, 2001). Injection of DAMGO, a selective MOR agonist, into the VTA decreases extracellular GABA release and increases the activity of DAergic cells (Narita et al, 2001). This mechanism is thought to underlie the high addiction rate caused by prescription opioids.

Although changes in enkephalin and endorphin signaling are not well established in chronic pain, it is known that opioids lose effectiveness in the long term and commonly result in tolerance issues (Dumas and Pollack, 2008). What mediates this exact mechanism is complex and not cemented, but it has been shown that the ability of opioids to stimulate DAergic neurons in the VTA is decreased in chronic pain (Taylor et al, 2016). Chronic pain decreases MOR activation in the VTA and this is tied to a reduction in opioid-induced DA release in the striatum (Ozaki et al, 2002). This mechanism translates physiologically: morphine, an exogenous MOR agonist commonly prescribed for severe pain, is unable to induce a conditioned place preference (CPP) in rodents with nerve injuries (Ozaki et al, 2002).

Given this, it seems likely that chronic pain induces the desensitization of MOR receptors, possibly mediated by internalization of the receptor, expressed on inhibitory VTA interneurons (Zuo 2005). This would result in an increase in GABA release and tonic inhibition of VTA DAergic projection neurons, explaining the pathological alterations present in the VTA of neuropathic animals. Given this information, dysregulation of MOR signaling in the VTA plays a probable role in altering mesocortical DA release in chronic pain states. However, MORs are not the only receptors in the brain demonstrated to mediate DA release.

The kappa opioid receptor (KOR) is recognized for its active role in gating the release of DA in the mesolimbic pathway (Xi et al, 1998). The peptide dynorphin is the endogenous agonist for KOR and dynorphin fibers have been shown to contact DA terminals expressing KOR (Svingos et al, 1999; van Bockstaele et al, 1995). G_i-coupled KOR are highly expressed on DA terminals in the mesolimbic pathway and their activation inhibits the release of DA in the NAc (Tejeda and Bonci, 2019). Systemic administration of KOR agonists is well-known for its aversive effect in both humans and rodents (Liu et al, 2019). The fact that selective ablation of KOR expression from DA neurons occludes KOR-induced aversion indicates the aversive properties of KOR agonists are likely mediated by their ability to decrease DA release (Liu et al, 2019).

Interestingly, KORs are also expressed on DA terminals in the prefrontal cortex and their pharmacological activation has been shown to disrupt cognition (Abraham et al, 2021). Furthermore, prodynorphin mRNA is increased in the ACC following nerve injury (Palmisano et al, 2019). Prodynorphin is the precursor to dynorphin and these results suggest that KOR over-activation on DAergic terminals in the ACC could be responsible for the decrease in DA levels following neuropathic pain. Further research is required to elucidate the role of KOR in mitigating DA release in the ACC and whether this system could be driving DAergic deficits in chronic pain.

In conclusion, both KOR and MOR signaling have been shown to influence the release of DA in the brain. As chronic pain appears to significantly impact both of these systems, they could be involved in dysregulated DA release in the mesocortical pathway, promoting pathological cortical hyperexcitability.

4.5 Pain relief requires D1R signaling in the ACC

Our results in Chapter 2 show that blocking pharmacologically D1Rs in the ACC occludes

gabapentin-induced CPP, indicating D1R signaling in the ACC is necessary for effective pain relief. These findings imply that removing the DAergic component, specifically in the ACC, of common analgesics such as gabapentin, lidocaine and opioids decreases their functionality. Opioids are known for preferentially decreasing the affective component of pain perception over the sensory component and low doses of opioids have been shown to promote DA release in the mesolimbic pathway (Oertel et al, 2008). Our results indicate that pain relief evoked by opioids, and other analgesics, could be predominantly mediated by their ability to facilitate the release of DA in the ACC. Further research is required to verify this theory, but if true, the implications could drastically transform the way opioids and other analgesics are prescribed to chronic pain patients.

4.5 Therapeutic Relevance

The results presented in Chapters 2 and 3 strongly support our hypothesis that a chronic pain-induced hypodopaminergic state contributes to the dysregulation of cortical circuits involved in top-down pain perception. We show that supplementing supraspinal DA with L-DOPA decreases pathological cortical excitability and associated symptoms. Given these results, should we start prescribing L-DOPA to chronic pain patients to treat symptoms of chronic pain? No, systemic treatments such as these tend to have an abundance of negative off-target effects and is exactly what we are trying to avoid (see side effects of systemic opioids). For most drugs, specificity is key and this is no different when testing the potency of analgesics such as L-DOPA. Chronic L-DOPA treatment induces cognitive deficits, muscle tremors, dyskinesia, and more (Godwin-Austen et al, 1969; Jenner et al, 2008). Nonetheless, a recent study demonstrated that small doses of L-DOPA are successful at alleviating chronic lower back pain, specifically in women (Reckziegel et al, 2019). Given this, treatment with low doses of L-DOPA might be a

viable strategy to treat select cases of chronic pain, but more selective therapies are still preferred. Alternatively, extremely low doses of L-DOPA coupled to other analgesics known to upregulate supraspinal DA release (e.g. opioids) could be a viable method to limit the side effects of L-DOPA as well as those of opioids. In this case, the interaction between the two could greatly increase cortical DAergic tone and decrease pain perception while limiting the unwanted side effects induced by greater doses of either L-DOPA or opioids.

Deep brain stimulation (DBS) of the VTA has been successful at treating chronic migraine pain (Akram et al, 2016). Although intrusive, since this technique has decreased effects on nigrostriatal DA transmission, less motor side effects are reported (Settell et al, 2017). Alternatively, if we can identify the genetically-distinct subset of DAergic neurons in VTA involved in modulating the pain circuits in the ACC, then gene therapy would add another layer of specificity and could become a potential viable option for chronic pain with little side effects. Although some of these subsets have been identified, specifically the DAergic neurons in the ventromedial VTA positive for $DAT^{+}/VGLUT2^{+}/CCK^{+}$ and dependent on Sonic HedgeHog signaling, this genetic therapy avenue requires much progress and development (Kabanova et al, 2015; Poulin et al, 2018).

As mentioned in the previous section, the cause of chronic pain-induced decreases in supraspinal signaling is still not fully understood. If identified, novel therapeutics could be aimed at preventing their onset rather than dealing with the associated symptoms. For example, if KOR overactivation in the ACC is a main mediator in promoting cortical hyperexcitability following nerve injury, then perhaps local administration of KOR antagonists could prove beneficial in the early stages of nerve injury. In fact, studies have already shown that administration of nor-BNI, a selective KOR antagonist, significantly decreases spontaneous pain in rodents (Lee et al, 2021).

In short, this dissertation identifies a susceptible mesocotical pathway affected by chronic pain that is heavily involved in mediating pain perception, but further understanding is still required to identify the most efficient way to exploit this pathway as a therapeutic option.

Limitations

4.6 Sex-specific differences

One of the greatest limitations of this work is the lack of research aimed at identifying sex-differences concerning the role of cortical DA in mediating pain perception. Pain perception is well known for its sex differences and sexual dimorphism in DAergic pathways has been reported, particularly in the spinal cord and the striatum (Megat et al; 2018; Mogil 2012; Munro et al, 2006; Pohjalainen et al, 1998). Although debated, the general consensus is that the DA system is enhanced in females (Zachry et al, 2021). This is mediated through a combination of: increase in tonic DA levels, differences in the distribution of midbrain DA neurons, and ovarian hormone regulation (Dewing et al, 2006; McArthur et al, 2007; Nolan et al, 2020). The ovarian hormone 17β-estradiol (E2) is particularly important to this sexual dimorphism as it has been shown to upregulate the activity of DA neurons and elicit DA release in the NAc and striatum (Yoest et al, 2018). Despite this, we have not investigated how chronic pain alters cortical DA release in female mice.

Furthermore, sex differences have been identified in DAergic signaling in spinal cord. A report indicates males rely primarily on D5R expression in the dorsal horn for descending pain modulation, whereas females rely on D1Rs (Megat et al, 2018).

These results are critically important as they could explain why L-DOPA was successfully at alleviating chronic lower back pain in female but not male patients (Reckziegel et al, 2019).

4.7 The innate problem with photometry

Although photometry is an invaluable tool for the investigation of brain circuits in freely-moving animals, its dependence on relative change rather than absolute values commonly leads to misinterpretations. Photometry analyzes the percent change in rapid fluorescence fluctuation and therefore is not useful at identifying long term alterations or determining changes in tonic expression. Given this, we are unable to determine if chronic pain leads to a tonic hypodopaminergic state in the ACC, independent of pain stimulation. Furthermore, if baseline levels of DA are altered, the dLight1.1 dF/F induced by reward or pain stimulation will not be accurately reflected. For example, assuming a normal 0.335 ± 0.025 pmol/ml extracellular concentration of DA in the PFC, the 5% change in dLight1.1 dF/F induced by pain indicates a 0.01675 pmol/ml decrease in extracellular DA (Bymaster et al 2002). Conversely, if extracellular DA is hypothetically decreased to 0.200 pmol/ml in chronic pain conditions, a 5% drop in fluorescence would indicate a decrease of 0.01 pmol/ml in extracellular DA. Therefore, although acute pain still reduces DA release in the PFC in chronic pain, the 67% decrease is undetectable using photometry. This effect is further compounded by the logarithmic relationship between DA concentrations and dLight1.1 fluorescence (Patriarchi et al, 2018).

Future Directions

This dissertation provides key insights into how DA influences the cortical circuits involved in pain perception, yet, further research is required to fully understand the role of not only DA, but other monoamines such as NE and 5-HT, in influencing these circuits.

Identify the interneuron subset(s) involved in D1R-mediated ACC GABA release

In Chapter 2 we identified that D1R activation in the ACC promotes the release of GABA on pyramidal cells. However, which neuronal population is involved in mediating this GABA release is not established. D1Rs are expressed on both parvalbumin (PV)- and somatostatin (SOM)-positive interneurons, the principal fast-spiking interneurons responsible for pyramidal inhibition in the neocortex (Muly et al, 1998). Determining which interneuron population is involved in D1R- mediated pyramidal inhibition can be readily accomplished by visually-guided electrophysiology recordings in brain slices of PV- or SOM-GFP transgenic mice. Optogenetic stimulation of ACC PV+ interneuron has been shown to increase nociceptive thresholds, so this inhibitory subset is particularly interesting (Kang et al, 2015).

Determine how chronic pain affects DA release evoked by reward and pain relief

This work explicitly looks at chronic pain-induced alterations in pain-evoked inhibition of DA release in the ACC. Reward and pain relief, known to promote the release of DA, are also likely affected by chronic pain. Chronic pain patients commonly report a decreased sensitivity to rewards, reduced motivation, and anhedonia. (Geha et al, 2014; Karoly et al, 2014; Munn et al, 2015). As all these symptoms are heavily linked to DA signaling, it is important to characterize

how chronic pain influences DA release evoked by reward, pain relief, and other experiences that promote DA release.

One study has found that pain relief-evoked DA release in the NAc is decreased in the late stages of chronic pain (Kato et al, 2016). Based on our results from Chapter 2, we know that pain relief also invokes DA release in the ACC, yet how this is impacted by neuropathic pain is still unknown.

Investigate changes in cortical release of NE and 5-HT in chronic pain

Given recent advances in GENIs selective for NE and 5-HT, the homeostatic mechanisms that regulate their release can now be investigated before and after the onset of chronic pain. As discussed in the literature review, these neuromodulators play a key role in modulating the ACC and the balance of 5-HT, NE, and DA release in the ACC is key to maintaining a healthy cortical excitability and normal sensory perception.

Explore the possibility and mechanisms of sex-specific differences

As previously mentioned, the lack of sex-specific analysis is a main limitation here. Clinical studies have demonstrated that L-DOPA is effective at reducing lower chronic back pain, specifically in females (Reckziegel et al, 2019). There are sex-specific differences in D1R and D5R expression in the spinal cord and this sexual dimorphism could be responsible for mediating the benefits of L-DOPA (Megat et al, 2018). Furthermore, the impact of estradiol on activity of DAergic cells in the VTA is undeniable and its role in mediating DA release in the ACC should be investigated (Yoest et al, 2018).

Conclusion

In conclusion, this dissertation establishes DA modulation of the ACC circuitry is critical in mediating the excitability of pyramidal neurons, is involved in descending top-down pain modulation, and its dysregulation in chronic pain states is likely promoting pathological pain states. Furthermore, we show that ACC D1R signaling is necessary for effective pain relief. Supplementation of supraspinal L-DOPA occludes pain relief induced by gabapentin, decreases pathological cortical excitability and associated symptoms in chronic pain. In line with these results, we demonstrate that the release of DA in the ACC is altered following the onset of chronic pain. Although more research is required to determine the molecular mechanism involved in chronic pain-induced decreases in supraspinal DAergic signaling, this is a step in the right direction.

I hope this dissertation asserts the idea that mesocortical DAergic signaling can be a viable therapeutic target for chronic pain patients and this novel knowledge promotes curiosity and consequently further investigation on the subject.

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